

A TEXTBOOK
of
BIOCHEMISTRY

Edited by

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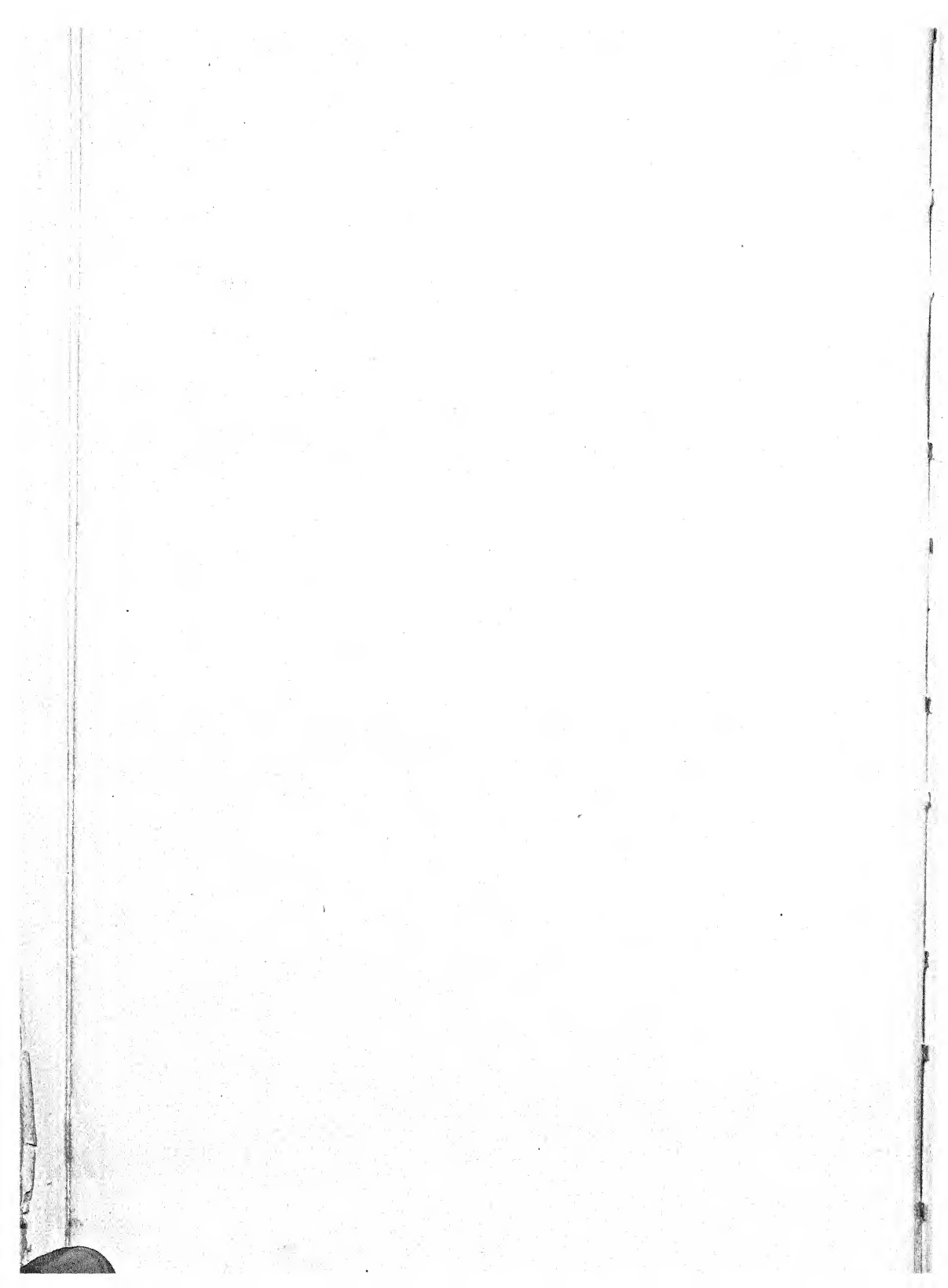
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PREFACE

BIOCHEMISTRY, like several other branches of science, has become so encyclopedic in its scope, that it seems an impossible task for any one individual to write an adequate textbook. It is for this reason that we have asked specialists in the various fields of biochemistry to contribute the chapters constituting this book. It is our hope that readers will find the treatment both authoritative and comprehensive.

Three chapters, not usually included in biochemical texts, have been added. The chapter on the cell, it seems to us, is as logical an introduction to biochemistry as a chapter on the atom would be to chemistry. The articles on immunochemistry and the chemistry of bacteria represent new and fertile fields for the expansion of knowledge.

This text, which has been planned for teachers and students of medicine, chemistry and allied branches, is sufficiently inclusive to fill the needs of a diversified group. Each instructor will obviously select for his group those portions of the text which he wants emphasized.

We have not hesitated to change, here and there, the customary sequence of chapters when, in our opinion, logical continuity demanded such a change.

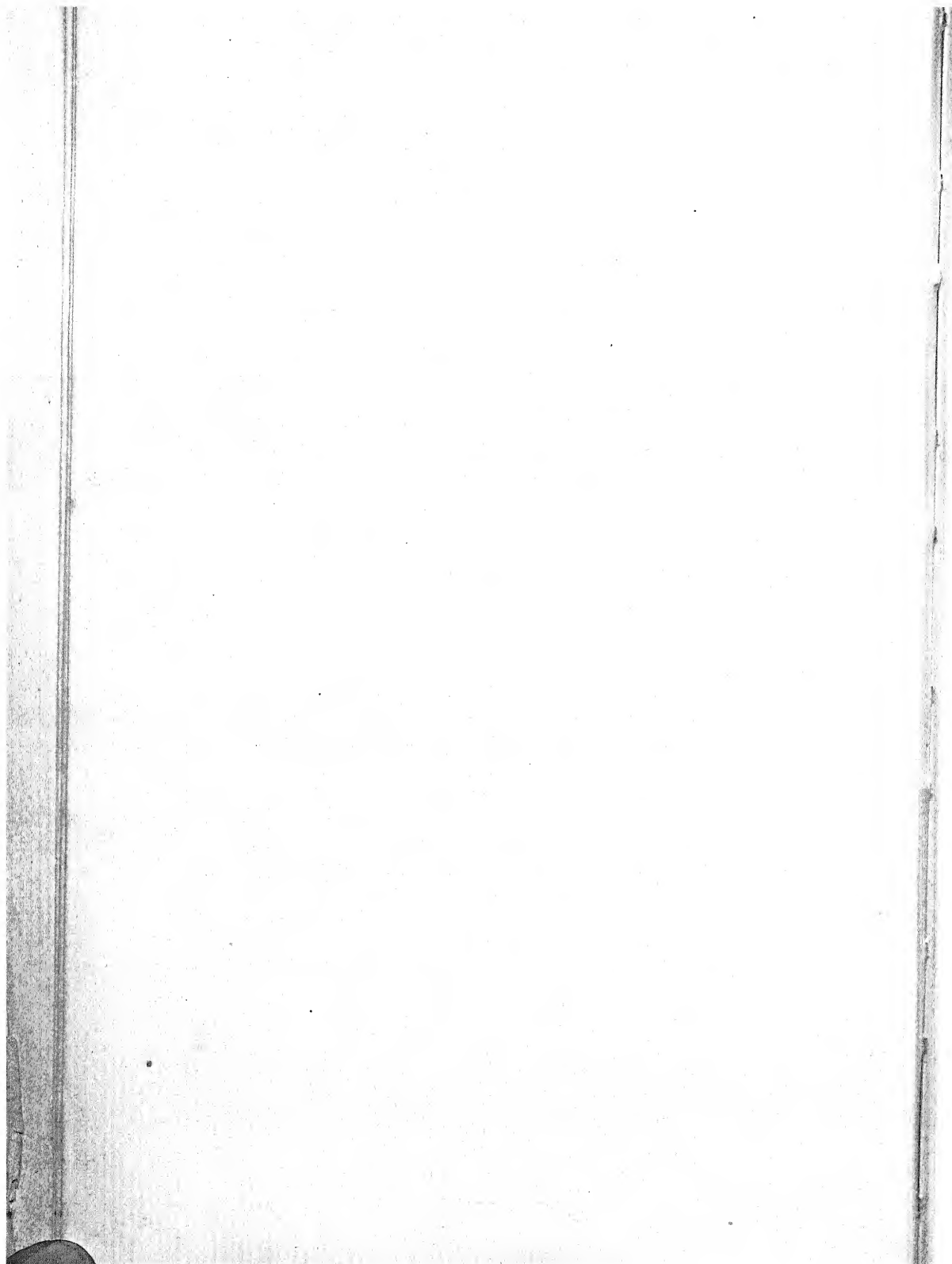
We can say very frankly that the task of editing this book was no task at all, but a source of great pleasure. We are among the first of its readers to have added to our stock of knowledge and to have gained a wider perspective.

Our thanks are due to our various contributors for their painstaking efforts in preparing worthwhile articles; and to W. B. Saunders Co., for their many helpful and practical suggestions. Dr. P. M. Apfelbaum has been good enough to proofread certain portions of the text. We have been helped in the checking of references by Mr. A. Mazur and Mr. E. Borek.

We trust that readers will not hesitate to call our attention to any imperfections, so that these may be avoided in a future edition.

NEW YORK CITY,
March, 1935.

BENJAMIN HARROW.
CARL P. SHERWIN.

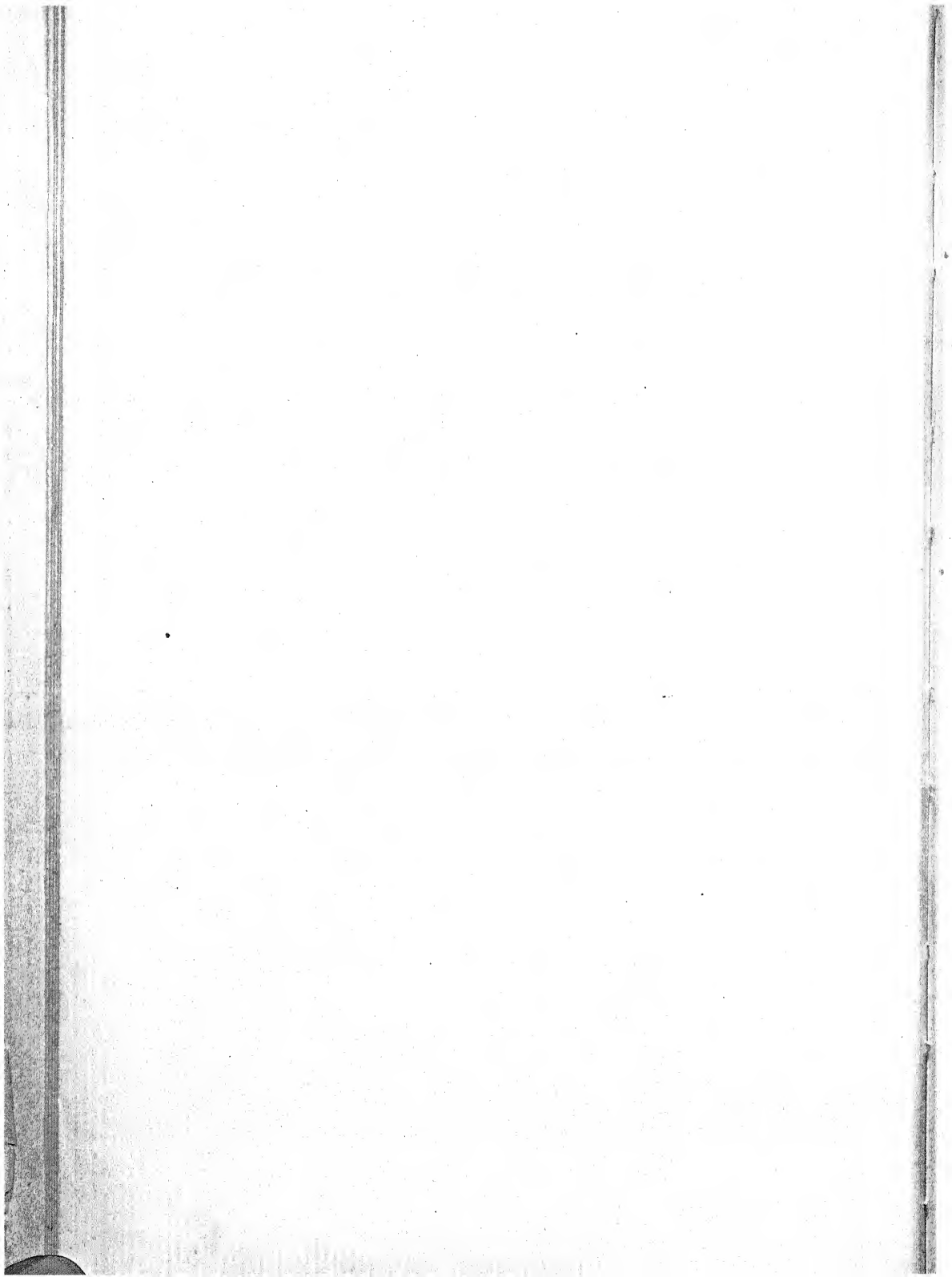


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Textbook of Biochemistry

CHAPTER I

THE LIVING CELL

CELLS are structural units of living matter, although in multicellular organisms they are subordinated functionally to the general economy of the organism as a whole. However, there is considerable evidence, especially clear from tissues grown *in vitro*, that the cells even of organized tissues, are able to exist as living units independently of the organism.

During the developmental period of a multicellular organism, the constituent cells undergo differentiations and become highly specialized in structure and function. The possibility of cellular dedifferentiation, *i. e.*, of reversion to a less specialized type, has been the object of much study. In tissue culture there is no clear evidence for its occurrence. An explanted fragment, *e. g.*, of heart or of gland, becomes disorganized as a tissue but the individual cells maintain their specificity. Even in neoplastic growths, where the cells lose many of their normal characteristics, there is no evidence that cells ever revert to a primitive, undifferentiated condition.

The protoplasm of the cell should be regarded not as a substance but as an organized, physicochemical system. In plant cells, which have prominent cell walls, the protoplasmic body is called the *protoplast*, a term which connotes an organized living unit and as such is preferable to the term "protoplasm" since the latter implies the more purely speculative aspects of living matter. A significant feature of the protoplast or protoplasmic body is its microscopical dimensions. In some large cells, *e. g.*, in *Valonia*, an alga, the size is due to the sap-filled vacuole; the protoplast which surrounds this vacuole may extend over a considerable area but only as a thin film not over several micra in depth. Likewise, in heavily yolk-laden eggs, such as the hen's egg, the protoplasm is concentrated in the minute germinal disk and in a thin film surrounding the yolk.

If the view is accepted that filtrable viruses and the bacteriophage are living, we would have to consider that the lower limit of living bodies approaches colloidal dimensions. This would bear out the micellar hypothesis in which the ultimate unit of living bodies is regarded as a micelle, possibly of enzymatic nature, and protoplasm an aggregate of living micellae. However, such a conception is at present

purely speculative. There are still many problems to engage our attention regarding the physical nature of visible cells which are soluble in terms of our grosser knowledge of physics and chemistry.

The aging of protoplasm is a question of great interest. A study of this in a single cell is complicated by periodic reconstructive changes which lead to the production of daughter cells the properties of which may differ from those of the parent cell. However, during the interval between two successive divisions, morphological changes have been noted as evidence that an aging process does occur. It has been found, for example, that the macronucleus of some protozoa increases in size disproportionately to the rest of the cell. This has suggested the idea that a change in the nucleus-plasma ratio is concerned in the initiation of cell division. It is doubtful whether such a conclusion can be generalized to the cells of the Metazoa.

Cell division does not always occur in constant rhythmic cycles. This is seen in many tissues in which the cells, after continuing for a long time without dividing, may suddenly, as a result of trauma, undergo a period of mitotic activity. This capacity for cell division seems to decrease with increased differentiation of the cells. An example of the importance of environmental factors is seen in tissue culture in which the multiplication of cells of adult tissues frequently can be enhanced by juices extracted from embryos when they will not multiply in their own fluids.

The length of time that a cell can live without undergoing cell division varies greatly from a few hours in many of the wandering cells of the body to the many years of a lifetime during which nerve cells are supposed to last. An interesting case, described by MacDougal, is that of certain desert cacti the cells of which grow without dividing for well beyond a century. MacDougal found that this growth was accompanied by a great increase in the size of the vacuole and by a wasting away of the protoplasm and by an increase of its permeability.

The question of structural continuity between contiguous cells has frequently been raised because of bridgelike connections which have been observed between the cells. By means of microdissection it has been possible to show that these connections between the deep cells of the human epidermis are actual, breakable strands. The significance of these bridges is little understood. In many organized tissues there seems to be no structural continuity between the protoplasts of contiguous cells.

Among the normally mononucleated cells of the body, cells are frequently found containing two or more nuclei. Examples of these are the giant cells in the spleen and bone marrow. Such cells have been supposed to arise from the fusion of several cells. It is equally possible, however, that they are caused by one or more nuclear divisions unaccompanied by cytoplasmic division. The greater likelihood of the latter view is suggested by the fact that the two halves of a cell about to undergo division can be kept from separating as long as the halves,

each with its own daughter nucleus, are still connected with a common stalk of cytoplasm. When once the stalk has broken through, fusion of somatic cells rarely, if ever, occurs. On the other hand, in syncytial, *i. e.*, multinuclear, organisms, such as the plasmodia of certain fungi and protozoa, fusion between separate individuals occurs frequently and spontaneously. An example of such a protozoan is the *Actinosphaerium* which may attain the size of a sphere of 0.2 to 0.3 mm. in diameter. Its protoplasm is coarsely vacuolated and can be cut readily into fragments which slowly round up. The spherical fragments readily fuse when brought into contact. A slow and constant flow of the protoplasmic material between the vacuoles gradually merges the two contiguous masses into a single body.

As a rule, cells cannot be considered to be isotropic. Even though their morphological structure may show no asymmetry they still may possess a functional polarity. An example of this is the *Valonia* which is a globular cell with a cellulose wall and a large vacuole of sap with a thin layer of protoplasm between the wall and the vacuole. It reproduces by budding and buds usually appear at the same end of the parent cell. A definite polarity has also been claimed for the seemingly amorphous ameba. Schaeffer¹ has found that some species of amebae move predominantly to the right, others to the left. When individuals are placed on a thin glass rod or within a capillary tube, the path is always either a left-handed or a right-handed helical spiral. The polarity appears to be variable, for Schaeffer found that certain external factors may modify the direction. For example, a normally left-turning species becomes strongly right-turning within thirty minutes after ingesting a large and easily digestible food object, and does not become predominantly left-turning again until several hours have elapsed. A change tends also to occur with increasing age and with exposure to certain intensities of light.

In a study of the physical constitution of protoplasm it is necessary to ascertain the extent to which protoplasm is normally enclosed within extraneous and nonliving coatings. In plant cells the protoplast is enclosed within a cell wall from which it can be detached and still remain alive. In animal cells the existence of an extraneous coating is less obvious, but nevertheless is probably an almost universal feature. Abramson² has shown that even leukocytes, which are usually classed as naked cells, behave in an electrical field as if they were covered with a film of protein. The difficulty in such a case is to know whether the protein-like coat is to be regarded as a nonessential covering or as a vital part of the protoplasm of the leukocyte.

One of the extensively studied and most controversial problems in cytology deals with the nature of the external boundary of protoplasm. Is the protoplast to be regarded so constituted that its property of selective permeability is to be ascribed to a differentiated surface layer, the so-called "plasma membrane"? Or is this property one of the entire mass of the protoplasm?

CELL MEMBRANES

Extraneous Coatings.—The readiness with which extraneous coatings develop on the surface of protoplasm and the fact that their existence has not been appreciated in many animal cells have been a source of confusion in ascertaining the physical nature of the naked protoplast.

In animal cells the existence of protein-like coatings is indicated by their reaction to various salt solutions. The presence of calcium salts tends to stiffen their surfaces and make them sticky, while sodium and potassium salts, in the absence of calcium, tend to soften and dissolve the material which holds cells together.

The protein-like coatings of animal cells and the cellulose walls of plant cells are to be regarded as products of secretion by the cells. For example, the blastomeres of marine echinoderm ova which have been denuded of their coatings by exposure to calcium-free sea water will acquire new coatings when returned to sea water with its full complement of salts. An analogous phenomenon has been described for plant cells, the protoplasts of which can be denuded by shrinking them from their walls by exposure to an hypertonic environment. In many instances the surface of the shrunken protoplast subsequently becomes coated with a new wall of cellulose.

The following experiment, done by the author (unpublished), demonstrates that protoplasm, denuded of its extraneous coating, still possesses a membrane which is not stiffened by the action of calcium salts and that this membrane is essential for the integrity of the protoplasm.

Mature, unfertilized sea urchin eggs are shaken vigorously in a quantity of sea water and then in an isotonic NaCl solution to rid them as far as possible of their envelopes. The eggs are now transferred to a drop of isotonic solution of CaCl_2 mounted on the under-surface of a coverslip which roofs a microdissecting moist chamber. Through the microscope many of the eggs will be seen stuck to the glass of the coverslip. The end of a blunt-tipped microneedle is then brought against one of the eggs and moved so as to push the egg ahead of the needle. The jelly-like material surrounding the egg holds fast to the coverslip but eventually breaks in one or more places; whereupon the naked protoplast of the egg can be made to slip out as if out of a shell. This naked egg rounds up immediately in the CaCl_2 solution. It is no longer sticky and can be rolled about freely. When pressed against the coverslip with the side of a microneedle it can be pinched into two portions each of which immediately assumes a spherical form. The egg behaves like an oil drop and the portions can be divided repeatedly into smaller, spherical droplets. The operation is successful only so long as the surface of the naked egg is not torn with the pointed tip of a needle. If this is done the tear immediately opens up and the interior becomes converted into a hard, frothy coagulum.

This experiment indicates that the sea urchin egg, after repeated washing, may still possess an investment which is stiffened and ren-

dered sticky in the presence of CaCl_2 . It also shows that this investment can be removed without destroying the protoplasm of the egg; and the reason the protoplasm persists is because of an additional investing membrane which prevents the CaCl_2 from penetrating into and coagulating the interior of the egg but which itself remains fluid in the presence of the salt.

The Plasma Membrane.—This term for the external surface layer of protoplasm was first used by Pfeffer, who postulated the existence of a semipermeable membrane to account for the results obtained in his classical studies on the osmotic properties of plant cells. Pfeffer and Nägeli, who preceded him, regarded the surface of protoplasm as a haptogen membrane, protein in nature.

Bütschli was opposed to the idea of a differentiated plasma membrane, holding that the integrity of a protoplasmic body is due to the immiscibility of its material with the aqueous environment. The absorption of water by protoplasm he regarded as analogous to the way in which watery fluids diffuse into fatty oils where they may collect as aqueous droplets. Lepeschkin³ has accumulated considerable data on this point, one of his chief arguments for Bütschli's view being that protoplasm can absorb water only to a limited degree. This he showed by exposing the protoplasm of marine foraminifera and of certain algae to diluted sea water and noting that the protoplasm of the cells, after swelling to a limited degree, became filled with vacuoles, the size and number of which varied with the dilution of the medium. When the cells were returned to ordinary sea water the vacuoles disappeared and the cells regained their normal appearance.

It is true that the penetrability into living cells of many substances, particularly of those which are lipoid-soluble, can be explained without postulating that a selective permeability is limited to a peripheral layer of the protoplasm. On the other hand, the membrane theory explains many more phenomena. One of these has already been mentioned, namely, the difference in the action of CaCl_2 on the surface and on the interior of the sea urchin egg.

Experiments on the electrical conductivity of cells offer another argument in favor of the idea that the external surface layer of protoplasm possesses properties decidedly different from those of the interior. These experiments were first done by Höber⁴ on red blood cells in serum and on frog's muscle in isotonic NaCl . Höber found that the impedance to the passage of a current decreases with a rise in the frequency of the alternating current and that at very high frequencies the impedance falls to a low value. These results have been confirmed by several investigators for mammalian tissues and for sea urchin eggs. The investigations indicate that the resistance to an electric current is principally due to the surface layer of a cell. This implies that ions cannot pass readily through the plasma membrane while they can move with comparative freedom within the internal protoplasm.

The relative impermeability of the surface of protoplasm as con-

trasted with its interior has also been shown by the micro-injection of various substances. Fresh water amebae when immersed in solutions of $MgCl_2$ at concentrations as high as one and two molar move about freely and show no injury. On the other hand, an injection of $M/50$ molar $MgCl_2$, of an amount less than the volume of the nucleus of the ameba, causes, at the site of the injection, an immediate coagulation which spreads until the entire ameba is involved. The effect of $CaCl_2$ is similar to that of $MgCl_2$ except for the complication that, unless the calcium salt is used in higher concentrations, the ameba immediately reacts by pinching off the region at the site of injection before the salt has had time to spread.

The diffusibility through the interior of protoplasm is most strikingly seen when injections are made of aqueous solutions of dyes which do not penetrate the cell. This is illustrated by injecting an aqueous solution of phenol red into a variety of cells such as living muscle fibers, gastric mucosa cells, starfish eggs and fresh-water amebae. If a microdroplet of the solution is injected into the cytoplasm the color quickly diffuses until it stops at the surface boundary. Evidently, the interior of the cytoplasm is freely permeable to the dye and the inability of the dye to get in from without or to pass out from within must be ascribed to the existence of a surface film or layer which is impermeable to the dye from either side. A great number of solutions have been injected into a variety of cells and in every instance a substance, which does not penetrate from without, will, when injected, diffuse through the interior.

Interesting cases are the injection of $NaHCO_3$ and of NH_4Cl into the cytoplasm of a starfish egg. It has long been known that CO_2 and NH_3 readily penetrate living cells while the highly dissociated HCl and $NaOH$ do not. Jacobs⁵ has shown that starfish eggs, immersed in an alkaline solution of $NaHCO_3$, develop an intracellular acidity while, on the other hand, an intracellular alkalinity occurs when the eggs are exposed to a solution of ammonium chloride. However, when these solutions are injected directly into the eggs the results are reversed. This is because the injection brings the entire solution into the interior of the cell with the result that the internal protoplasm reacts to the alkalinity of the carbonate and to the acidity of the ammonium chloride solutions.

Thickness of the Plasma Membrane.—Under dark ground illumination a bright line frequently is visible at the boundary of the protoplasm and some investigators have ascribed a measurable thickness to it. However, it is highly probable that such a measurement includes more than the actual plasma membrane responsible for the relative impermeability of the protoplasm.

Injection experiments indicate that the thickness of the plasma membrane is far too slight to be visible. If, for example, an ameba or a starfish egg is immersed in a solution of a dye which cannot penetrate and if a quantity of the same solution is injected into the cell there is

no evidence of a colorless zone between the color inside and outside the cell. From electrical conductivity experiments the thickness of this membrane has been estimated to be of molecular dimensions.

The very fluid state of the plasma membrane can be demonstrated in the following manner with a starfish egg. The extraneous coating of an egg is torn and a part of the protoplasm allowed to flow out as an exovate. With a delicate microneedle the naked surface of the exovate is seized and dragged out for some distance in the form of a filament. On reversing the movement of the needle the substance of the filament flows back into the exovate with no sign of wrinkling on its surface. If the filament is stretched farther it may break into a string of coherent droplets. On lessening the tension the droplets flow together and when the filament is released it flows back into the main body of the protoplasm.

Formation and Repair of the Plasma Membrane.—The protoplasm of a living cell is able to repair a torn surface if the tear is not too extensive, and if the environment is normal. This is well illustrated in microdissection experiments on starfish eggs in sea water. No breakdown occurs if the tip of the needle is moved slowly through the protoplasm and out of its surface. But if the piercing action is performed suddenly the surface film disrupts and the exposed cytoplasm will begin to pour out and its granules to scatter in the medium. While this is occurring, films may appear around the masses of the disorganizing material which swell and burst. Frequently films form within what appears to be normal cytoplasm. These films may unite and reach the intact film which surrounds the remaining portion of the egg after which no further disintegration occurs.

In the normal environment of sea water the repair is too rapid to permit the penetration of a dye to which the egg is normally impermeable. In slightly acidulated water, the repair is slower and the dye enters presumably because the dye has had time to get in before the impeding film has been sufficiently reconstituted.

The Action of Salts on the Maintenance of the Plasma Membrane.—Calcium salts enhance the formation of the plasma membrane while sodium and potassium salts tend to do the reverse. This reaction must not be confused with the action of these salts on the extraneous coatings of cells which are stiffened and coagulated by calcium. Calcium in the medium tends to stabilize the plasma membrane but, at the same time, to increase its fluidity. This is well shown in the experiment on naked sea urchin eggs. A striking analogy regarding the effect of Ca and Na on soap films is given by Clowes.⁶

Attempts have been made to apply a solution of calcium chloride to the inner surface of the plasma membrane of the sea urchin egg. A micropipet was inserted through the interior, and the solution expelled when the tip of the pipet was close to the inner side of the far surface. The result was always a coagulation so that friable strips could be pulled from the surface with a needle. However, it is impos-

sible to ascertain whether the injected CaCl_2 coagulates the plasma membrane by attacking it from the inside or whether the coagulation is due to the action on the cytoplasmic material underlying it.

The effect of NaCl on the plasma membrane is very different from that of CaCl_2 . This is shown when the surface of echinoderm eggs is torn in an isotonic solution of NaCl after the extraneous coats have been removed. Starting at the tear, a wave of disintegration travels over the surface of the protoplasm and converts the film into minute droplets, whereupon the internal protoplasm flows out and becomes dissipated in the medium.

The conditions for the maintenance and repair of the plasma membrane are at an optimum in a solution containing both NaCl and CaCl_2 in the proportion existing in sea water. In such a solution or in sea water the protoplasm of these eggs can be pulled into long cylinders and cut into spherules with no evidence of cytolysis.

THE INTERNAL PROTOPLASM

The Consistency of Protoplasm.—The consistency of the internal cytoplasm varies considerably. In many plant protoplasts its fluidity is evident from the continuous streaming movements which it exhibits in cyclosis. In animal cells it is viscid and semisolid in some cases and liquid in others. In very fluid protoplasts, plant and animal, there is often a more solid cortex of varying thickness lying directly beneath the plasma membrane.

These differences in consistency have been ascertained by various methods, one of which is to observe the effect of centrifugal force in dislocating the visible constituents within the cell. This method has shown that the viscosity of protoplasm even in the same cell is not constant; also that variations in the temperature of the environment affect the viscosity. Heilbrunn⁷ has found, for example, that the internal viscosity of the egg of the clam, *Cumingia*, is at a maximum at about 15°C . On each side of this temperature the viscosity drops to a minimum at about 2°C . and at 30°C . At 1°C . and at 31°C . there is a sudden and pronounced increase in the viscosity. He states that this increase in viscosity differs from the heat coagulation of proteins in being reversible. It is possible that the changes in viscosity are related to changes in the permeability of the cell to water at the various temperatures and to changes in the state of the fatty materials within the cell.

The existence of brownian movement, made visible with dark ground illumination, appears to be almost a universal phenomenon in protoplasm. This movement can be observed not only in the more fluid regions of cells but also in regions which, by means of microdissecting needles, have been shown to be in a jellied state.

A remarkable feature is the fact that semisolid regions in protoplasm can sometimes be made suddenly fluid by mechanical agitation. This phenomenon can be exhibited in a sea urchin egg undergoing di-

vision. At this time the fluid protoplasm is being converted into two jelly-like, semisolid masses. If a microneedle is inserted into one of the masses and the needle is suddenly jerked, the mass becomes fluid. If the needle is then removed or allowed to remain stationary for a few minutes, the original jellied condition returns and the division of the egg proceeds normally.

It is noteworthy that the normal activities of a cell may continue even when the fluidity of its protoplasm is increased by exposing the cell to hypotonic conditions. This can be seen in fertilized sea urchin eggs immersed in sea water diluted with distilled water to as much as two thirds of its original concentration. Under such conditions the protoplasm becomes so fluid that the visible granules within it undergo violent oscillatory movements evident even under ordinary transmitted illumination. In spite of this, nuclear division takes place and the egg segments in the usual way.

The Water Miscibility of Protein.—Micro-injection experiments present strong evidence that water forms a continuous phase in protoplasm. Aqueous solutions diffuse readily through the cytoplasm of such diverse types of cells as protoplasts of root-hair cells, amebae, various ova, muscle fibers, nerve cells, and ciliated epithelial cells. To insure success the injection must be performed gradually, otherwise a localized disintegration may occur. In the latter case the resulting injury may spread and produce complete cytolysis. Frequently, however, a membrane may form around the disintegrating region to constitute a vacuole while the rest of the protoplasm remains unaffected.

Water-immiscible fluids, when injected, always form discrete droplets, the shape of which is conditioned by the presence or absence of structural elements in the cytoplasm. For example, in the ameba a droplet of olive oil may assume a spherical shape while in the muscle fiber of the frog it is compressed into a cylindrical shape.

The water within the cytoplasm freezes readily.⁸ To demonstrate this, the interior of the subcooled cell must be inoculated with an ice crystal since the external membrane tends to obstruct the spread of freezing from outside the cell. In the ameba the freezing is in the form of feathery ice crystals which spread in all directions from the site of the inoculation. In the case of the muscle fiber of a frog the ice forms long, slender columns between denser longitudinally arranged constituents of the fiber. If the frozen condition is maintained for more than a few minutes the columns progressively enlarge and the dehydration of the protoplasm becomes irreversible.

The Hydrogen Ion Concentration of Protoplasm.—Since protoplasm is an organized structure which is destroyed by crushing, it is obvious that a determination of the hydrogen ion concentration of extracts of crushed tissue cannot be a reliable clue to the intensity of acidity within the living cell. Moreover, the unavoidable admixture of intercellular fluid from the tissue introduces an additional source of error.

Reliable results can be obtained only while the protoplasm is living. Electrometric measurements with micro-electrodes inserted into living cells have not yet been successful. Colorimetric methods, which have afforded fairly consistent results, fall into three groups: The use of natural indicators already existing in cells, vital staining with dyes having a pH virage, and the micro-injection of indicators into the protoplasm.

Natural indicators have been unreliable mainly because they are usually localized in vacuoles which do not necessarily have the same pH as the surrounding cytoplasm. Vital staining is open to the same objection since the vital stains tend to be segregated in vacuoles.

The micro-injection of lipid-insoluble indicators into the protoplasm has given results least open to question.⁹ Even then the color tends to be more or less rapidly segregated in vacuoles. Values obtained for the intracytoplasmic pH by the best available methods lie between 6.8 and 7.2. These values have been obtained by injecting a series of indicators of overlapping ranges into such cells as echinoderm eggs, various somatic cells of vertebrates, the protoplasts of plant cell and of amoebae. By this method it has also been found that cytoplasmic vacuoles give color indications which have no necessary relation to the observed pH of the surrounding cytoplasm. In Metazoa the cell nucleus has been found to be consistently more alkaline than the cytoplasm, the colorimetric value from injected indicators being in the neighborhood of pH 7.6 to 7.8.

Both cytoplasm and nucleus maintain their respective pH values even when the pH of the environment is shifted by means of penetrating acids and bases. The pH of the cytoplasmic vacuoles, however, changes readily. It was by the color change of neutral red in these vacuoles that the penetration of ammonia and of carbon dioxide was first determined. The buffering power of the protoplasm seems to be limited, and the pH can, in some cells, be upset to the extent of several tenths of a pH unit on either side of the normal pH value. Death ensues, however, when the pH is thrown too far in either direction, particularly to the acid side. Mechanical injury which initiates cytolysis, even when localized, is accompanied by the production of acidity, the intensity of which is in the neighborhood of pH 5 to 5.5. On the other hand, the nucleus, when injured, gives no evidence of changed pH .

In short, we find within the living cell the remarkable phenomenon of regions which are circumscribed by well-defined membranes and differ in the intensity of the acidity: The nucleus with a pH of 7.6 to 7.8, the cytoplasm with a pH of 6.8 to 7.2, and the cytoplasmic vacuoles with a variable pH which can be shifted readily by penetrating acids and alkalis in the medium.

During the metabolic activity of cells the production of acid has no effect on the nuclear or cytoplasmic pH , but it may have a pronounced effect on the pH of the vacuoles and of the medium surrounding the cells. The latter is well shown in muscle where an observable

shift in pH by the production of acid occurs only in the interstitial fluid between the muscle fibers and never within them. Evidently, acids produced within the protoplasm are immediately buffered so that the appearance of free acid occurs only at the boundaries outside the cytoplasmic system.

The Action of Salts on the Internal Protoplasm.—The relatively neutral reaction of the internal protoplasm suggests that much of the protein present is on the alkaline side of the iso-electric point. This is borne out by the ease with which coagulation occurs when salts of polyvalent cations, even in very dilute concentrations, are injected into such cells as echinoderm eggs, amebae, and the protoplasts of plant cells. For example, CaCl_2 , in all concentrations down to $M/200$ and HgCl_2 in very low concentrations produce a coagulating effect which is either restricted or extensive according to the amount injected. The injection of NaCl and of KCl has no such effect, presumably because they form soluble salts of the protein present.

An additional argument for the existence of proteins on the alkaline side of their iso-electric point in the protoplasm is offered by the fact that picric acid, when injected into a cell, exerts no coagulating action as long as the internal protoplasm maintains its normal neutral reaction. Pollack found that it was possible to inject a saturated aqueous solution of picric acid into an ameba with no other effect than to color it a vivid yellow. The significance of this experiment (unpublished) is brought out by injecting a mixture of picric acid with a solution of brom cresol purple, an indicator which is yellow in acid solutions below pH 6 and purple in more alkaline solutions. When a moderate amount of the bright, yellow mixture is injected the ameba assumes a murky green color and remains alive. When too much is injected the color remains yellow and the ameba becomes coagulated and dies. These facts are explained on the basis that with moderate injections the proteins remain on the alkaline side of their iso-electric points and the picric acid is converted into the ineffective sodium picrate. However, if the amount injected is large the excess acid carries the proteins to the acid side of their iso-electric points thus permitting the formation of an insoluble protein picrate.

The ease with which the internal protoplasm is coagulated by CaCl_2 in contrast to the noncoagulating effect of NaCl and of KCl has been questioned by Heilbrunn⁷ who obtained opposite results by immersing sea urchin eggs in solutions of NaCl and CaCl_2 isotonic with sea water. Physical changes observed within a cell from immersion experiments are difficult to interpret since they may not always be due to penetration of substances from the medium. However, even if the effect is assumed to be due to penetration, the difference in results still may be accounted for by the difference in the amounts of the salts which are brought into the cell by the two methods of experimentation. The difference in the effect of injection and of immersion is brought out strikingly if instead of CaCl_2 we use the salt of a basic dye, *e. g.*,

neutral red. This dye is a vital stain and, if present in the medium in appropriate dilutions, will readily penetrate cells and color their cytoplasmic granules and vacuoles without any sign of injuring the cell. However, if an aqueous solution of the dye is injected directly into a cell, the protoplasm at the site of injection is converted immediately into a red, coagulated mass. Apparently, in the usual method of vital staining by immersion the dye is taken up by vacuoles and granules as fast as it penetrates and before it has time to combine with the cytoplasmic proteins, although coagulation may even then occur if an excessive amount of the dye salt is present in the medium.

STRUCTURES IN PROTOPLASM

Membranous structures are of great functional importance in protoplasm. The plasma membrane which maintains the integrity of the protoplasm has already been discussed. Another membrane is that which bounds the nucleus. Its permeability seems to be quite different from that of the plasma membrane. For example, by micro-injection experiments, it has been shown that the nuclear membrane is freely permeable to sublethal concentrations of HCl while the plasma membrane is not. In addition to these membranes are the ones which surround the cytoplasmic vacuoles. All three types of membranes are easily destroyed when a solution of NaCl is blown against them.

Cytoplasmic vacuoles often appear *de novo* and their contents are usually aqueous. The factors determining their appearance are not known. It is difficult to imagine how a vacuole with aqueous contents and surrounded by a nonaqueous film can form in cytoplasm, the continuous phase of which is almost certainly aqueous. Not only do they form *de novo* but they also can increase in size and can concentrate solutes within them.

Granules, as such, are probably not as frequent as has been supposed since most of the so-called "granules" are actually minute vacuoles. Examples of such structures are the spherical pigment granules of the sea urchin egg. They average somewhat less than 2 micra in diameter and when they are punctured with a microneedle the investing membrane breaks and the pigment diffuses out. However, rod-shaped and irregular granules also do exist and appear to be more or less solid in texture. Fatty and crystalline bodies are also known to be present but they most frequently appear enclosed within vacuoles.

In the so-called "honey-combed" protoplasm, characteristic of some animal cells, especially protozoa, there are numerous alveoli which give a foamy appearance to the protoplasm. The injection of aqueous solutions into protoplasm of this type¹⁰ sometimes presents a possible exception to the view that water can diffuse easily through the substance of protoplasm. This is only apparent, however, and not real. The inserted tip of the pipet usually pierces the wall of an alveolus and the injected solution passes directly into the alveolus where it is retained locally for a time.

In the ameba and in many other protozoa there are digestive and contractile vacuoles. Indicator solutions, introduced into the digestive vacuoles, show progressive changes in the acidity of the contents. The contractile vacuole is supposed to be concerned with the elimination of water. It is known to accumulate some salts; *e. g.*, sodium picrate.

The mitochondria are minute, globular or filamentous structures which appear to be universally present. They are vitally stained with Janus green and are probably lipoid in nature. Unlike the vacuoles and alveoli they can be torn by microneedles without losing their identity. Their significance is not known.

The nature of another structure, the "Golgi apparatus," has been a matter of considerable controversy. This structure is prominent in glandular cells. In the living cell it has been described as a group of localized vacuoles more or less intimately related to certain mitochondria. In fixed and stained material it usually appears as an irregular meshwork which is specifically stained with silver salts by reduction of the silver. Its presence in glandular cells is of particular interest and suggests a structural polarity in cells which are known to absorb materials from one side and to deliver products of secretion at the other.

Still another structure which may be mentioned is an ill-defined area called the "sphere" or "center" lying in the cytoplasm close to the nucleus. Wherever it has been recognized in cells, it has always been found to take a prominent part in the division of the cell. It is the first visible structure to divide. The resulting two daughter spheres then move apart until they lie at opposite poles of the cell with the nucleus between them. It is significant that the events which now take place are concerned with a general movement of materials to and their accumulation in the vicinity of the spheres. Because of this, the spheres are frequently called attraction spheres or attraction centers.

The nucleus is well recognized as an essential constituent of protoplasm, and the morphology of the chromosomes which appear in it in cells preparing to divide has been the object of exhaustive study mainly in regard to the property assigned to them of being bearers of hereditary characteristics. Physically the chromosomes are gelatinous bodies which appear in the nucleus by the shortening of tenuous filaments into relatively compact and dense bodies shortly before the nuclear membrane disappears and preparatory to nuclear and cell division. It is not known whether or not the filaments preexist in the nucleus as morphological structures during the period between successive nuclear divisions.

Little has been accomplished in a study of the physical nature of the nucleus in the living state, chiefly because it is very susceptible to mechanical injury. The cytoplasmic portion of a living cell can be punctured and torn with microneedles and recovery is still possible even when the tear has been so drastic as to induce partial cytolysis.

On the other hand, a slight tearing of the nucleus rapidly destroys not only the nucleus but also the surrounding cytoplasm.

In many cells, particularly the Metazoa, the nucleus is an easily deformable, fluid spherule bounded by a membrane. An example of a fluid nucleus is that of the immature starfish egg in which the nucleoli can be observed to fall merely under the influence of gravity. In some cells the nucleus reacts to needles as if it were an elastic jelly and in others it seems to be a liquid with a pronounced tendency to change into a stiff jelly when manipulated and torn.

The nucleus of the immature starfish egg remains liquid after puncturing so that it can be injected with solutions. The injection of NaCl does not affect the fluid state but an injection of CaCl_2 causes coagulation. Aqueous solutions of basic dyes, *e. g.*, neutral red, upon injection, rapidly diffuse into the surrounding cytoplasm, while solutions of acid dyes, *e. g.*, phenol red, remain within the nucleus. This is probably due not to the relative impermeability of the nuclear membrane but to the greater degree of alkalinity of the nuclear interior in contrast to that of the cytoplasm.

That the nucleus is essential to the life of the cell has long been appreciated from the fact that nonnucleated fragments of a cell do not survive long. This may be due in part to the lack of the division mechanism which, as a rule, is considered to be the impetus to renewed cell growth. Nonnucleated fragments of amoebae move and ingest food but they do not seem to be able to complete the process of digestion. Nonnucleated fragments of plant protoplasts, at least of some species, have been described as being able to develop new cellulose walls around them but they do not seem to be capable of growing in size.¹¹

Probably the most direct evidence of the importance of the nucleus is seen when one of the nuclei of a binucleated cell is punctured.¹² Tearing the nucleus of a mononucleated cell always results in the death of the cell. On the other hand, if the cell possesses two nuclei the injurious effect of puncturing one nucleus may be counteracted by the presence of the other nucleus. This is shown in the following experiment: One of the nuclei of a binucleated fibroblast in tissue culture was pricked with a sharply pointed microneedle. The punctured nucleus collapsed and the extended pseudopodia of the cell were withdrawn. The cytoplasm in the vicinity then became highly granular while the filamentous mitochondria degenerated into spherules. The region around the other nucleus, however, showed no signs of degenerating. Five minutes later, pseudopodia extended again from the region which had been affected, the excessive granulation disappeared and, within ten minutes after the puncture of the one nucleus, the cell was again completely expanded and had regained its former aspect, except that now it appeared as a typical, mononucleated cell.

PERMEABILITY

Methods of Studying Permeability.—One of the most useful methods for investigating the physical constitution of protoplasm has been through permeability studies. The conclusions drawn from these studies are inferred from a knowledge of the nature of the substances which are found to penetrate or not to penetrate. A great variety of substances have been used many of which are foreign to the cells. It is also necessary to experiment with as many different types of cells as possible. The greatest difficulty in interpreting the results lies in the possibility of unsuspected alterations in the properties of the cells during the experimental procedure.

An extensively used method, especially with plant cells, is the plasmolytic one which depends upon the fact that protoplasm readily undergoes osmotic volume changes. When a substance to be tested is added to a medium containing plasmolyzed cells the penetrability of the substance is determined by the extent and rate with which the shrunken protoplasts regain their original dimensions, *i. e.*, undergo deplasmolysis. A frequent source of error in this method is the possibility of unsuspected leakage of solutes from the cells during the experimental procedure and also the possibility that osmotically active substances may be generated by hydrolysis of solutes within the cell. Another method for determining permeability is to observe detectable changes within the cells subsequent to their immersion in various solutions. Good results have been obtained by using dyes which betray their presence within cells by their color. Of considerable significance are those dyes which change color according to their lipid and water solubilities and to their degrees of dissociation at different hydrogen ion concentrations. Still another method for studying permeability is to immerse a quantity of cells in a solution of a given substance and, after a time, to remove the cells by centrifuging and determining analytically the amount of the substance remaining in the solution. The mass of cells is also analyzed chemically to ascertain the amount they may have absorbed from the medium. This method is uncertain because substances may be removed from the medium not by having penetrated the cells but by having adhered to their external surfaces.

The most direct method, and the one seemingly least open to question, is to use single cells which are large enough so that chemical analyses of the cell interior can be made after the cell has been immersed in a solution of a given substance. This has been possible with certain plant cells the protoplasts of which enclose vacuoles containing watery sap in sufficient amounts for micro-analysis. Examples of these are the long, narrow, multinucleated cells of *Nitella* and *Chara* and the globular *Valonia* cells which can be obtained in sizes from a small pea to almost a hen's egg. Experiments on these plant cells have afforded valuable data as to which substances can pass through the protoplasm and collect within the enclosed vacuoles. However, since the analyses are of the vacuolar sap only, they offer no data

regarding the possibility that certain substances may pass through the plasma membrane into the protoplasm without going through it into the vacuole. That this does occur is indicated by the following experiment with methyl red, an amphoteric dye:¹³ In solutions within the physiologic pH range (6-8) it preferentially accumulates in the most alkaline solution. If a plant cell is immersed in a solution of methyl red at pH 6 the dye will progressively accumulate in the more alkaline cytoplasm of the protoplast, while the vacuole which has a pH about 5.8 remains colorless. If a solution of the dye is injected into the vacuole the dye is immediately taken up by the protoplast until the vacuole becomes colorless, and the dye will not escape from the cell so long as the medium is kept at a pH of about 6 or less.

Overton's Theory.—An adequate discussion of results obtained in permeability studies is beyond the scope of this chapter. The problem is complicated not only because of the varied interpretations which have been offered but because, in the light of more recent investigations, many of the recorded observations must be considered as incomplete. The earlier investigators and, indeed, many today have been too prone to regard the cell as a sac in a static equilibrium with its environment. Even the term "permeability" connotes passiveness. The property of a living cell to absorb and reject materials undoubtedly depends upon a continuous expenditure of energy and is capable of pronounced readjustments to changes of environmental conditions.

The problem can best be appreciated by a brief review of the work of Overton, who established an experimental basis for a conception of permeability which, except for its somewhat static viewpoint, is as acceptable as any known today.^{4, 5, 14}

Overton compared the penetration of a large number of dyes into living cells (plant cells, blood corpuscles, muscle cells) with the solubility of the dyes in solutions of lipids in organic solvents. The solution which most closely approximated the living cell in taking up the dyes from water he found to be cholesterol or lecithin dissolved in xylol or benzene. The greater the amount of cholesterol or lecithin present the closer was the approximation. This observation, and the fact that lecithin and cholesterol appear to be present universally in living cells, suggested to him that the specific permeability of cells depends upon a surface layer consisting mainly of cholesterol or of lecithin-like substances.

Overton also used the plasmolytic method to determine the penetration of many organic compounds. Among the alcohols he found that penetrability decreased with an increase in the number of OH groups; *e. g.*, ethyl alcohol penetrated much more rapidly than did ethylene glycol. He also found that specific atomic grouping affected the penetrability; *e. g.*, acetamide with the same number of carbon atoms as ethyl alcohol penetrated much more slowly than the alcohol. Moreover, in a homologous series of the two groups the penetrability increased with the length of the carbon chains. Overton correlated these

results with the partition coefficients of the substances concerned between aqueous and nonaqueous solvents and concluded that those factors which favor solubility in organic solvents are also the ones which tend to favor penetration into cells.

Jacobs⁵ has pointed out that almost all of Overton's results conform to the rule that the ability of a given organic compound to enter a living cell is increased by reducing the number of polar groups within the molecule. In his experiments with dyes Overton confined himself, as a rule, to the salts of the dyes in neutral solutions. However, he was aware of the fact that alkaloids penetrate more readily as bases than as salts. This empirical discovery he explained on the ground of the greater lipid solubility of the basic form, a fact which, in years subsequent to his investigations, has been found to depend upon electrolytic dissociation which is determined by the pH of the medium. An example of this is the striking experiment in which Overton found that fish or tadpoles survived for a time in a solution of one part of strychnine nitrate to 1000 parts water; but if a small quantity of sodium bicarbonate was added they quickly succumbed. Overton explained this by stating that the increased alkalinity of the medium converted the nonpenetrating lipid-insoluble alkaloid salt into the rapidly penetrating lipid-soluble and therefore highly toxic alkaloid base.

Overton did not extend his experiments to ascertain whether there might be a slow penetration of typical, lipid-insoluble solutes. Investigations with such substances have necessitated modifications of Overton's relatively simple hypothesis that penetration is due merely to solubility in lipoids.

Permeability to Water.—The ease with which water can pass into and out of cells is clearly shown by the fact they readily shrink or swell according to whether the medium is hyper- or hypotonic to the cells. The compatibility of this fact with the assumption that the plasma membrane is lipoidal has already been indicated.

The effect of electrolytes on the permeability of cells to water can be interpreted in terms of the dispersive or stabilizing action of the various electrolytes on the plasma membrane. Lucké and McCutcheon¹⁵ immersed sea urchin eggs in hypotonic solutions of dextrose containing electrolytes in concentrations too small to alter appreciably the osmotic pressure. They found that the chlorides of Ca and of Mg decreased the rate of swelling in the hypotonic dextrose, while the chlorides of Na, K, Li and NH_4 caused the eggs to swell as rapidly as, or even more rapidly than, in the hypotonic dextrose solution alone.

It has been stated that various narcotics, *e. g.*, methyl and ethyl alcohol, and chloroform, decrease the permeability of cells to water. For example, R. S. Lillie found that sea urchin eggs which shrink in a given hypertonic sea water will shrink more slowly if alcohol is added.⁵

Permeability to Nonelectrolytes.—As a general rule living cells are readily permeable to lipid-soluble nonelectrolytes irrespective of their molecular dimensions. A necessary condition, however, is that such

nonelectrolytes which do penetrate must also possess an appreciable degree of water solubility. The reason for this is not only because the nonelectrolyte must pass through an aqueous medium to reach the cell but also because it must be able to diffuse through an aqueous phase within the protoplasm of the cell. Micro-injection experiments on the ameba offer strong evidence that not only is water a continuous phase in the internal protoplasm but that the nonaqueous phase is discontinuous. It is known that the lower members of the hydrocarbon series exert a narcotic effect which is in direct proportion to their solubility in water. When these hydrocarbons are dissolved in olive oil their partition coefficient between the oil and water is such that the amount passing into the water is reduced to inappreciable quantities. With this relation in mind, Marsland,¹⁶ has shown that the injection of a droplet of the hydrocarbon, decane, into the interior of an ameba will cause narcosis but that the injection of a droplet of olive oil containing decane in solution has no such effect. If, however, a droplet of the decane-olive oil mixture is brought into intimate contact with the plasma membrane of the ameba, narcosis soon occurs. Evidently the seat of the narcotic action is mainly in the plasma membrane. The experiment also suggests that only in the plasma membrane does there exist a continuous lipid phase in which the hydrocarbon can spread.

Regarding the purely water-soluble nonelectrolytes, it is significant that not only is the rate of penetration very slow, but the actual penetrability seems to be related to molecular size. Collander and Bär-lund¹⁷ have recently determined the penetration of a large number of nonelectrolytes into the cells of *Chara* by collecting and analyzing the sap from the vacuoles of single cells after immersing them in the various solutions. The substances included various alcohols, acid amides, alkyl citrates, glycols, glycerols, ureas and sugars. Their penetration was found to increase with their solubility in ethyl ether or in olive oil. In close agreement with the above are experiments performed by Höfler¹⁴ on the cells of *Majanthenum* and by Jacobs and Stewart¹⁸ on sea urchin eggs. An interesting deviation from the usual technic is the recent work of Amberson and Höber¹⁹ who obtained essentially similar results by perfusing the salivary gland of the cat with hemoglobinized physiologic salt solution containing various nonelectrolytes and collecting the saliva, which was caused to flow by stimulating the chorda tympani nerve.

It is significant that some of the substances studied by Collander and Bär-lund penetrated more readily than would be expected from their oil solubility, *viz.*, formamide, acetamide and ethylene glycol. All these are characterized by small molecular size. Others which were relatively less penetrating than their oil solubility would warrant had larger molecular dimensions. Evidently, therefore, the molecular size of nonelectrolytes plays a rôle in the process.

Permeability to Electrolytes.—In regard to the penetrability of salts the situation is considerably more complicated and the question

arises whether protoplasm can be considered to be permeable also to ions.

Salts as a rule appear to penetrate living cells with considerable difficulty. An apparent exception to this is the case of the ammonium salts of fatty acids which Stewart²⁰ has shown effect cells as if they penetrated with ease. However, she has pointed out that these salts hydrolyze into ammonia and free fatty acid which are largely undissociated. Stewart found that *Arbacia* eggs, immersed in sea water containing these ammonium salts, underwent swelling, and the rate of swelling increased with the increase in length of the carbon chain of the fatty acid used, *viz.*, formic < propionic < butyric < valeric. This is also the order of their lipoid solubility. Stewart suggests that the ammonia and fatty acid when once inside unite to form the salt. This effect together with the lipoid-soluble acid and base would increase the internal osmotic pressure and the cell would swell. An interesting corollary to this is Jacobs' finding that *Arbacia* eggs *do not* swell appreciably in isotonic solutions of ammonium chloride the hydrolytic constituents of which are ammonia and hydrochloric acid. It is well known that HCl, in viable concentrations, does not penetrate living cells, and the ammonia, having a considerable affinity for HCl, would not accumulate within the egg sufficiently to exert an appreciable osmotic effect.

Other investigations, however, have indicated that ionized salts,¹⁴ at least those of penetrating acids²¹ do penetrate. Howard²² investigated this problem on *Arbacia* eggs using sodium salts of the saturated fatty acids. As a criterion for penetration of the fatty acids she used the liquefying effect determined by centrifuging. This she found to be more pronounced with each increase in concentration of the acid. By varying the concentration of the corresponding salt and keeping the amount of the free acid constant, Howard noted a decrease in the liquefying effect with increased concentration of the salt. She concluded that the liquefying action of the fatty acid was being antagonized by the presence of increasing amounts of the salt of the fatty acid. This would seem to agree with the conclusions of Smith¹⁴ that the salts of penetrating acids are able to penetrate. As an alternative to Smith's conclusion Howard suggests that the presence of the salt in the cell might be accounted for by the penetration of the acid and a subsequent cation exchange (the internal H^+ for an external cation). This idea was advanced on the assumption that the plasma membrane is permeable to cations but not to anions. The concentrations used in the experiments were very low, and Howard could observe no swelling of the eggs which presumably should result if the salt were accumulating within the egg.

Another paper which presents evidence for the greater penetrability of the salt of a weak acid from a solution of the acid and the salt is that of M. M. Brooks.²³ She determined, by direct analysis, the amount of arsenic in the vacuole of *Valonia* after exposure of the cell

to varying concentrations of arsenious and arsenic acids to which were added varying amounts of NaOH to obtain values of pH from 5 to 9. The analysis was made for the total arsenic in the sap, the amount obtained being compared with the concentration of the undissociated acid outside. Brooks found that the accumulation of arsenic in the sap increased with the increase in concentration of the ionized sodium salt in the environment.

The transport of ions across the plasma membrane is not difficult to interpret on the basis of the membrane being lipoidal in nature, if we assume the existence in the membrane of organic salts with which ionic interchange may take place. It is to be remembered that Bütschli obtained the accumulation of water drops in olive oil when the oil was rancid or contained oleic acid, and that Overton likened the plasma membrane to a lecithin or cholesterol mixture in an organic solvent. Possibly a closer analogy, proposed by Nirenstein (*cf.* 4, 5, 14) is a solution of oleic acid and diamylamine in olive oil. Variations in the amount and nature of the organic acid and basic constituents in the membrane would account for the varying permeability of different types of cells to the ions of inorganic acid and basic salts.

PENETRABILITY BASED ON CELLULAR ACTIVITY

Active Incorporation of Solutes.—Overton, to whom we owe the establishment of the lipid membrane theory of permeability, states that his theory was intended to account only for passive penetration by solubility. He was fully aware of its inadequacy for all phenomena of permeability.

Within recent years it has been recognized that living cells never reach a state of equilibrium with their environment but rather maintain a "steady state" which involves a continuous expenditure of energy derived from metabolic processes; further, that the permeability properties of living cells are affected by changes in their metabolic activity.

Cells differ in their ability to accumulate salts from their environment. Some selectively accumulate potassium while others accumulate sodium. Osterhout and Stanley²⁴ have simulated this behavior by a model consisting of two aqueous phases separated by a non-aqueous phase. One of the aqueous phases (representing the medium in which a cell is immersed) contains KOH and NaOH; the other (the "cell sap") is water through which a stream of CO_2 is bubbled. The nonaqueous phase (the "protoplasm") is a mixture of guaiacol and paracresol. In this model there occurs a continuous accumulation of K and Na in the "sap" so long as the CO_2 is present in that phase in excess.

"Physiologic" permeability is a term applied by Höber to cases in which certain substances, which are known to be nonpenetrating to most cells, have, nevertheless, under special circumstances been found to penetrate some cells. One striking case, discussed by Höber, is the penetration of phenol red into *Opalina*, a protozoon, parasitic in frogs.

Phenol red is a lipid-insoluble sulfonated compound which, in neutral or weakly alkaline solutions, exists as the highly dissociated Na salt and will not penetrate such diverse cells as echinoderm ova, amebae, muscle fibers and various plant cells. Höber found that the Opalinae likewise are impermeable to phenol red if the organisms are removed from the frog and placed in a physiologic saline solution containing phenol red. On the other hand, if the dye is introduced into the cloaca of the frog the Opalinae present quickly become colored an orange yellow. Höber has suggested that this is due to specialized physiologic activity of the Opalinae. In support of this is the observation of Hertz, cited by Höber, that narcotics hinder the entrance of lipid-insoluble dyes without affecting the penetration of lipid-soluble dyes.

The cells of the proximal segment of renal tubules are likewise exceptional in their permeability to phenol red. In the normal state, at body temperature and with adequate oxygen supply, a special permeability mechanism exists which appears to be unidirectional. The dye is taken up by the cells of the wall and is passed through and into the lumen of the tubule. Under the action of some oxidation inhibitors the cells lose their ability of transporting the dye in this way. The experimental finding that the direction of permeability in the cells of the renal epithelium can be reversibly affected by oxidation inhibitors is another approach to the problem of ascertaining the determining causes of variations in permeability. This raises the interesting possibility that the existence of a functional polarity in cells may be related to oxidative processes since, when metabolism is lowered and the respiratory mechanism is inhibited, the cell, although still alive, is unable to function as a polarized mechanism.

The existence within a single cell of regions possessing differences in concentration of oxidases and dehydrogenases has so far not been demonstrated. Indications with reducible dyes have not been conclusive. The existence of such regions might account for directed gradients of function in the cell which are obliterated by anaerobic conditions.

Active Incorporation of Particulate Matter.—Many of the wandering cells of the body are able to phagocytize particles with which they come in contact. This property is exhibited also by some epithelial cells. The problem has been studied mostly with ameboid cells which do not seem to discriminate between such objects as starch grains and iron or carbon particles. The phenomenon has been accounted for on the basis of surface tension conditions which cause the object being ingested to adhere to and be wetted by the protoplasm. A familiar illustration of this idea is the ingestion of a shellac-coated rod of glass by a drop of chloroform. The problem has been extensively studied with leukocytes²⁵ and it is generally agreed that surface tension as a factor must be decidedly a subordinate one. Fenn has shown that adhesiveness and phagocytosis do not go hand in hand; for example, in hypotonic sodium chloride solution the stickiness of leuko-

cytes to carbon particles is markedly increased while the ability to ingest is decreased, and the reverse is found to be true when the medium consists of blood serum. The optimum temperature for phagocytosis is 37° C., while adhesiveness is frequently greater at 20° C. The experiments made by Mudd indicate that the phagocytosis of bacteria by leukocytes is largely conditioned by a "wetting" of the bacterium by the leukocyte.

The process of ingestion in the comparatively large fresh-water ameba is easy to follow. In this organism the facts do not support the postulate that wetting must precede ingestion. It is possible to observe a wetting of the surface of the ameba with various oils. This has been shown by Dawson and Belkin²⁶ by an experiment in which a pipet with a drop of olive oil on its tip was pushed firmly against the ameba so that the oil came into intimate contact with the surface of the ameba. In many instances this operation caused the droplet suddenly to snap on and to assume a surface of contact which was concave with respect to the ameba. No ingestion took place and the ameba moved about with the "cap" of oil always in front. This phenomenon occurs with droplets of all sizes. Dawson and Belkin also have shown that a droplet of the same oil can be ingested by the ameba provided the ameba extends pseudopodia around the oil drop without adhering to it. The food cup thus produced closes over the object to form a food vacuole. The formation of food vacuoles by amebae has been extensively studied by Schaeffer.¹ He has described the extraordinary fact that amebae may sometimes be incited by the presence of a particle to form a food cup which closes over without even incorporating the particle.

A somewhat similar phenomenon of incupping of the surface has been described by Lewis²⁷ for leukocytes. For the cases recorded Lewis has used the term "pinocytosis" in which the surface of the leukocytes forms minute invaginations which close to form clear, fluid-filled vacuoles.

ROBERT CHAMBERS.

REFERENCES

1. Schaeffer, A. A.: *Ameboid Movement* (1920).
2. Abramson, H.: *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. 1 (1933).
3. Lepeschkin, W. W.: *Kolloidchemie des Protoplasmas* (1924).
4. Höber, R.: *Physikalische Chemie der Zelle und der Gewebe* (1924).
5. Jacobs, M. H.: *General Cytology* (1924) (ed. E. V. Cowdry).
6. Clowes, G. H. A.: *J. Phys. Chem.*, 20, 407 (1916).
7. Heilbrunn, L. V.: *The Colloid Chemistry of Protoplasm* (1928).
8. Chambers, R., and Hale, H. P.: *Proc. Roy. Soc. (London)*, 110B, 336 (1932).
9. Chambers, R.: *Bull. Nat. Research Council*, No. 69, 37 (1929).
10. Chambers, R., and Howland, R. B.: *Protoplasma*, 11, 1 (1930).
11. Küster, E.: *Pathologie der Pflanzenzelle* (1929).
12. Chambers, R., and Fell, H. B.: *Proc. Roy. Soc. (London)*, 109B, 381 (1931).
13. Chambers, R., and Kerr, T.: *J. Cell. and Comp. Physiol.*, 2, 105 (1932).
14. Gellhorn, E.: *Das Permeabilitätsproblem* (1929).
15. Lucké, B., and McCutcheon, M.: *Physiol. Rev.*, 12, 68 (1932).
16. Marsland, D.: *J. Cell. and Comp. Physiol.*, 4, 9 (1933).

17. Collander, R., und Bärlund, H.: *Acta Bot. Fennica*, **11**, 5 (1933).
18. Jacobs, M. H., and Stewart, D. R.: *J. Cell. and Comp. Physiol.*, **1**, 71 (1932).
19. Amberson, W. R., and Höber, R.: *J. Cell. and Comp. Physiol.*, **2**, 201 (1932).
20. Stewart, D. R.: *Biol. Bull.*, **60**, 171 (1931).
21. Smith, H. W.: *cf. Gellhorn* (14).
22. Howard, E.: *Biol. Bull.*, **60**, 132 (1931).
23. Brooks, M. M.: *cf. Gellhorn* (14).
24. Osterhout, W. J. V., and Stanley, W. M.: *J. Gen. Physiol.*, **15**, 667 (1932).
25. Mudd, S., McCutcheon, N., and Lucké, B.: *Physiol. Rev.*, **14**, 210 (1934).
26. Dawson, J. A., and Belkin, M.: *Biol. Bull.*, **56**, 80 (1929).
27. Lewis, W. H.: *Johns Hopkins Hosp. Bull.*, **49**, 17 (1931).

[The results of studies on microdissection and injection, for which no references have been cited in this chapter, are either hitherto unpublished or appear in the following books: Cowdry, E. V., *General Cytology* (1924); Alexander, J.: *Colloid Chemistry* (1928), Vol. II; Beutner, R.: *Physical Chemistry of Living Tissues and Life Processes* (1933).]

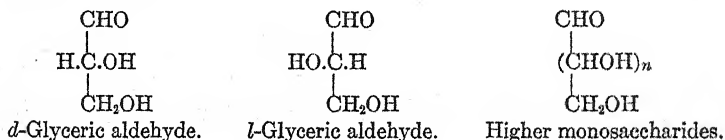
CHAPTER II

THE CARBOHYDRATES

THE SUGARS

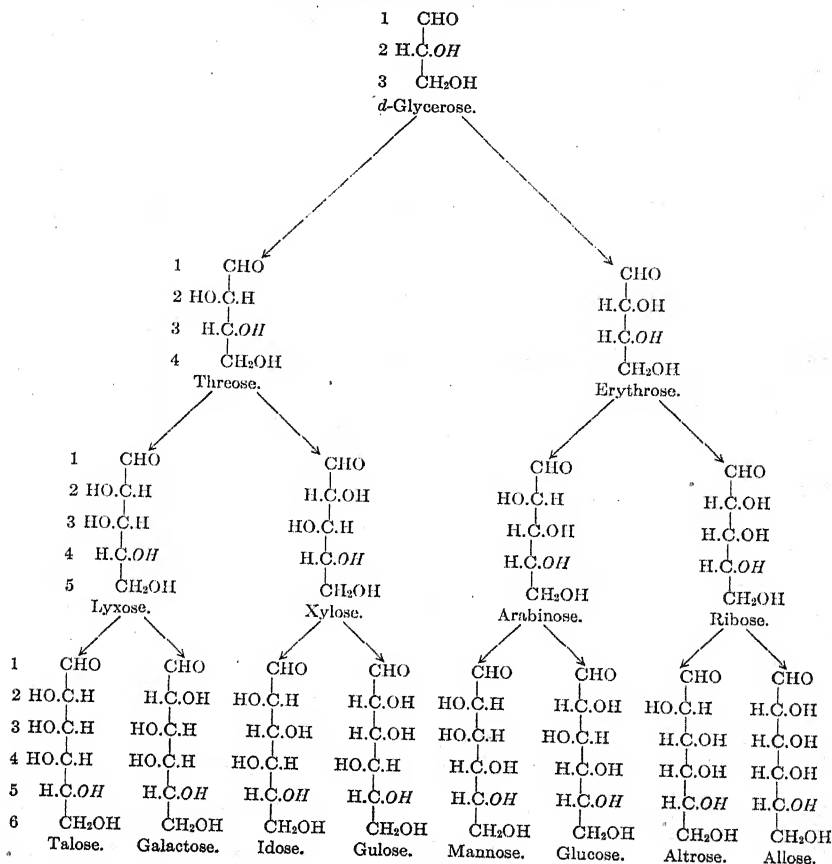
THE simple sugars or monosaccharides belong to the group of substances known as carbohydrates, the term originating in the fact that their elementary composition may be expressed by the general formula CH_2O . They are colorless, crystalline substances which have a more or less pronounced sweet taste.

The simplest known substance which, in many of its properties, resembles a sugar is glycollic aldehyde (diose) $\begin{array}{c} \text{H}-\text{C}=\text{O} \\ | \\ \text{H}_2\text{C}-\text{OH} \end{array}$, and the substance is frequently referred to as the lowest member of the sugar series. However, all natural sugars are optically active, which means that they contain at least one asymmetric carbon atom, whereas glycollic aldehyde possesses none. Hence it is more proper to regard glyceric aldehyde as the simplest sugar, the parent substance of all the higher sugars.



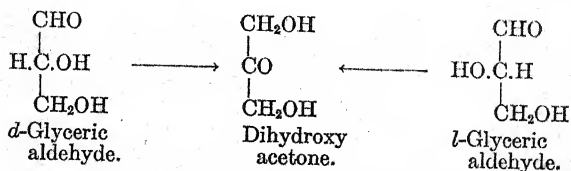
The monosaccharides are classified according to the number of carbon atoms in the chain (*i. e.*, dioses, trioses, tetroses, pentoses, hexoses, etc.). In glyceric aldehyde there is only one asymmetric carbon atom and therefore its structure permits only two optically active isomers—one dextrorotatory, the other levorotatory. In the higher members the number of asymmetric centers is determined by the number (*n*) of (CHOH) groups, and the number of active forms of a sugar is given by van't Hoff's expression 2^n . The higher sugars derived from dextro-glyceric aldehyde are classified in the *d*-series and those derived from levo-glyceric aldehyde are classified in the *l*-series. Each sugar, by acquiring an additional asymmetric carbon atom, leads to two isomeric (epimeric) sugars of the next higher order. A chart of the aldoses derived from *d*-glyceric aldehyde and constituting the *d*-series, is shown in the table on page 41.

The relationship of all these sugars derived from dextro-glyceric aldehyde is determined by the fact that they possess the identical configuration of the asymmetric carbon atom farthest from the reduc-

CONFIGURATION OF THE *d*-ALDOSES

ing group, the sugars of the *d*-series having the hydroxyl of the penultimate carbon atom to the right. In the *l*-series the configuration of the carbon atom adjacent to the primary alcoholic group is opposite to that given in the above projection formulae. This building up of the higher sugars from the lower is not only a theoretical possibility, but has actually been accomplished by a method introduced by Kiliani and specially developed by E. Fischer (see p. 48).

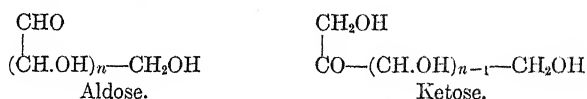
A further property of glyceric aldehyde, in common with other aldehydic or *aldose* sugars, is its power of isomerization to a keto structure or *ketose*.



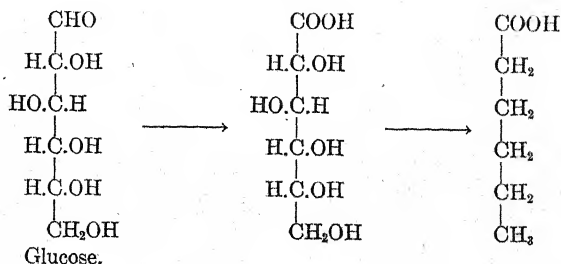
This isomerization causes one carbon atom to lose its asymmetry, so that both glyceric aldehydes lead to the same dihydroxyacetone. Similarly, each pair of aldoses epimeric with respect to carbon atom 2 leads to one ketose; hence the total number of possible keto-sugars is only half that of the aldo-sugars (as shown in the following table):

| Monoses. | | Number of asymmetric carbon atoms (n). | Number of stereo-isomers (2^n). |
|----------|----------|---|--|
| Aldoses. | Ketoses. | | |
| Diose | Triose | 0 | 1 |
| Trioses | Tetroses | 1 | 2 |
| Tetroses | Pentoses | 2 | 4 |
| Pentoses | Hexoses | 3 | 8 |
| Hexoses | Heptoses | 4 | 16 |

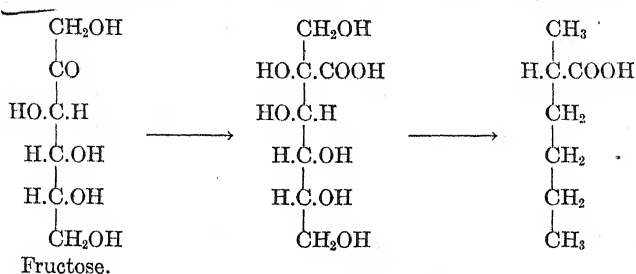
The general formulae for an aldose and its corresponding ketose may thus be written:



Structure of the Monosaccharides.—The structural formulae shown on page 41 have been assigned to the sugars by reason of their power to display properties common to carbinols on the one hand, and to aldehydes (or ketones) on the other. These properties will later be discussed in detail. Here it suffices to state that, as in ordinary alcohols, each hydroxyl group can be reduced by the action of hydriodic acid to a hydrogen atom; and the aldo- or keto-sugars should lead to simple aldehydes or ketones. Unfortunately, the process of reduction with hydriodic acid is a very drastic reaction, and the entire sugar molecule suffers disintegration under this treatment. It was therefore found expedient first to convert the aldose sugars into the corresponding sugar acids and to reduce the latter. Thus, in the case of glucose, oxidation gives the corresponding pentahydroxycaproic acid (gluconic acid), which may be reduced to normal caproic acid:



In the case of fructose, the keto-isomeric form of glucose, oxidation breaks the carbon chain, but the keto-sugar may be converted into an acid having an additional carbon atom (through the cyanhydrin reaction) and reduction of this leads to 2-methyl-n-caproic acid:



These reactions furnish proof that in the sugars presented on page 41 the carbon chain is a normal aliphatic chain and that all oxygen atoms but one can be accounted for by hydroxyl groups. It must be borne in mind, however, that sugars containing a branched carbon chain are also possible and have, indeed, been found in nature.

The Cyclic Structure of the Free Sugars.—Although the above straight-chain structure of sugars had been the one generally accepted, Tollens¹ as far back as 1883 suggested for them a cyclic structure (through the formation of an inner half-acetal; see p. 46). The basis for this conclusion was the fact that in certain respects the sugars do not behave as ordinary aldehydes or ketones. Thus, they do not give Schiff's test, nor do they form a stable addition product with sodium bisulfite; and they react with hydrocyanic acid only in the presence of some alkali. Although the arguments of Tollens were ignored, even by E. Fischer for a time, certain later discoveries made acceptance of the cyclic formulation imperative. The phenomenon which first attracted attention to this problem was that of mutarotation.

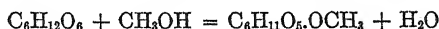
Mutarotation.—In 1846 Du Brunfaut found that freshly crystallized glucose has a specific rotation of $+111^\circ$ in water, but if the aqueous solution is left to stand the rotation diminishes to $+52^\circ$. He termed the phenomenon *birotation* because the rotation was approximately halved. In the course of time other sugars were examined, and it was found that the rotation is not necessarily halved, so the phenomenon was named *multirotation*. In 1899 Lowry² showed this to be also a misnomer and rechristened the phenomenon *mutarotation*, which merely means "change of rotation." The change is catalyzed by a trace of acid or alkali, the speed being increased though the final value of the specific rotation is, of course, unchanged.

In 1879 Landolt suggested that the change might be attributed to slow breakdown of crystal aggregates to molecules. Arrhenius showed this to be untenable as there is no change in the molecular weight. In 1890 Fischer likened the change to the hydration of a lactone and suggested that it was reversible. This is not correct, since mutarotation

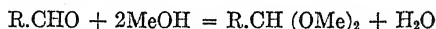
of substituted sugars in which the reducing group is free proceeds in dry solvents like chloroform and formamide.

In 1896 Tanret³ dissolved ordinary α -glucose (specific rotation $+111^\circ$) in boiling pyridine and on cooling he obtained β -glucose (specific rotation $+19^\circ$). On dissolution in water, the specific rotation of both forms changes slowly to the equilibrium rotation ($+52^\circ$).

Glycoside Formation.—When Fischer⁴ examined the condensation of certain of the reducing sugars with boiling methyl alcohol (in the presence of a small concentration of hydrogen chloride, which catalyzes the reaction), he found that the reducing function was lost and the sugar acquired a methoxyl group. For instance, with a hexose:



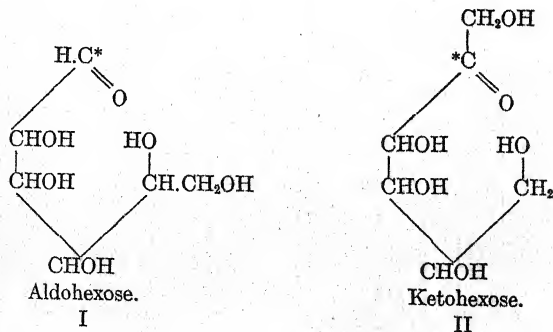
It is clear that the reaction involves the elimination of the elements of water and is to be considered a condensation. The addition is thus $=CH_2$, and not $=OMe_2$ as would be expected did the reaction involve ordinary acetal formation, as in the familiar reaction between an alcohol and an aldehyde:



From the reaction product Fischer was able, in certain cases, to isolate two stereo-isomeric substances from each sugar. The generic name now applied to this group of nonreducing substances is *methylglycoside*, the two forms being designated α and β respectively.

The formation of two such isomers from any one sugar can only be explained by assigning to them a heterocyclic ring structure.

As already indicated, the projection formulae afford but an inadequate means of representation either of the distribution of the hydroxyl groups in a sugar or of its chemical and physical properties. A strong tendency to ring formation is to be expected of such chain compounds on theoretical grounds. Owing to the tetrahedral angle between adjoining carbon valencies, the atom model of a sugar resembles a ring more than it does a straight chain, and the proximity of the aldehydic group* to the fourth (γ) and fifth (δ) carbon atoms, or of the ketonic group* to the fifth and sixth carbon atoms, is evident (formulae I and II).



It is therefore highly significant that glyceric aldehyde, in which there is no γ -carbon atom, possesses all the typical aldehydic properties; and that dihydroxy acetone acts as a true ketone, giving a stable condensation product with sodium bisulfite. On the other hand, the tetroses, in which a γ -carbon atom makes its first appearance, have lost some of the typical aldehydic properties.

Since the only way of explaining the appearance of the extra asymmetric carbon atom is to adopt for the glycosides a heterocyclic ring structure, the question arises as to whether this is a 3-, 4-, 5- or 6-membered ring. The methods by which this problem has been solved are discussed later.

Besides the methylglycosides there are many other sugar derivatives which exist in α - and β -stereo-isomeric modifications. Thus, for glucose the following are but a few of the derivatives which are known in both the α - and β -forms: Pentacetyl glucose, tetracetyl methylglucoside, tetracetyl nitroglucose, tetracetyl glucose, tetramethyl methylglucoside. Most of them are interconvertible and some exhibit mutarotation, yielding an equilibrium mixture of constant rotation.

After a cyclic structure had been definitely established for the glycosides, the logical step was to extend the same formulation to the parent sugars. To Simon belongs the credit for discerning this analogy. It remained for Armstrong to demonstrate that hydrolysis of α -methylglucoside with maltase led to the initial formation of α -glucose, and the hydrolysis of β -methylglucoside with emulsin to the formation of β -form (II) (see p. 46).

| | | | |
|--------------------------------|------------------------------|------------------------|------------------------------|
| α -Methylglucoside..... | $[\alpha]_D^{20} +157^\circ$ | α -Glucose..... | $[\alpha]_D^{20} +111^\circ$ |
| β -Methylglucoside..... | -33° | β -Glucose..... | $+19^\circ$ |

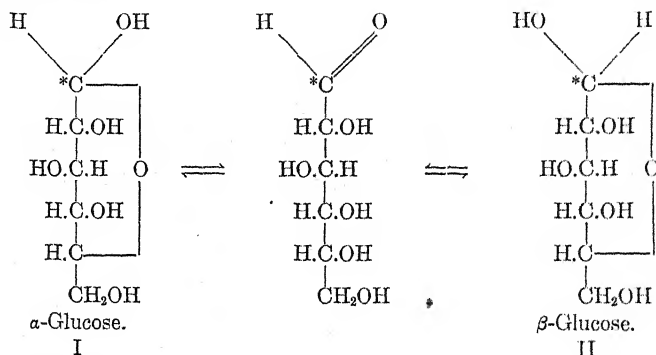
Two isomers, known as the α - and β -forms, appear in place of each single aldehydic or ketonic form. They are structurally identical and differ only in the disposition of the hydroxyl on the first carbon atom,* which is directed to the right in the α -form (I) and to the left in the β -form (II) (see p. 46).

In both the sugars and glycosides the two forms (α - and β -) have been arbitrarily named, by virtue of their different optical rotatory powers, according to Hudson's rule:¹¹ The names should be so selected that for all sugars which are genetically related to *d*-glucose the subtraction of the rotation of the β -form from that of the α -form gives a positive difference, and for all sugars which are genetically related to *l*-glucose an equal negative difference.

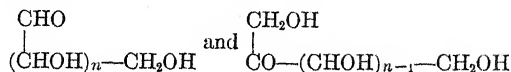
The *cis* or *trans* disposition of the hydroxyl groups attached to carbon atoms 1 and 2 has been revealed by an ingenious method devised by Böeseken, who has shown that the electrical conductivity of boric acid is increased by substances containing *cis* hydroxyls. The substance known as α -glucose caused a rise in conductivity and so is assigned the *cis* structure. Obviously, four of the α -*d*-aldohexoses—glucose, galactose, allose and gulose—have *cis* hydroxyls on carbon

atoms 1 and 2, and the other four—altrose, idose, mannose and talose—have *trans* groupings in these positions.

Lowry² showed the change in rotatory power to be a process of reversible isomeric change. Hence, it is now considered that the aldehydic form of the free sugar represents only an intermediate phase between the two cyclic α - and β -forms which exist side by side in aqueous solution. It follows that mutarotation may be due to (a) direct oscillation of the OH and H on the first carbon atom from one side of the chain to the other, or (b) formation of the "straight" chain aldehyde as an intermediate.

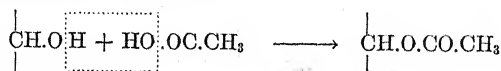


Alcoholic Properties.—The aldoses and ketoses have respectively the general formulae

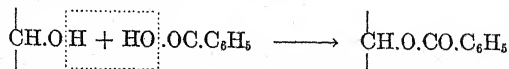


and contain, in addition to the reducing group, either one or two primary alcoholic groups and one or more secondary hydroxyl groups. Just as with the simple carbinols, these hydroxyl groups are capable of condensing (with the elimination of a molecule of water) with acids to form esters, with alcohols to form ethers and with aldehydes or ketones to form acetals.

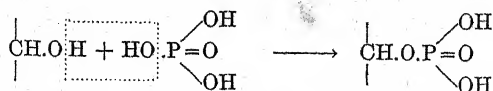
Formation of Esters.—Thus, with acetic acid the sugars may be visualized as forming acetates:



with benzoic acid, benzoates:

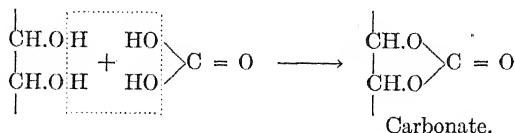


and with phosphoric acid, phosphates:

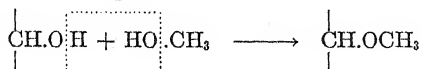


These reactions are generally performed by treating the sugar with the appropriate acid chloride (or anhydride) in the presence of cold pyridine (which neutralizes the hydrogen chloride liberated).

Chelate esters (borates, carbonates and ortho-acetates), in which the acid radical engages two adjacent *cis* hydroxyl groups, are also known.



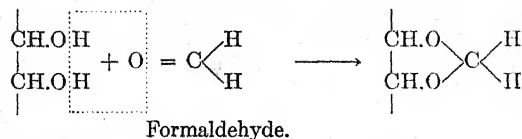
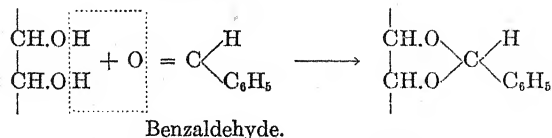
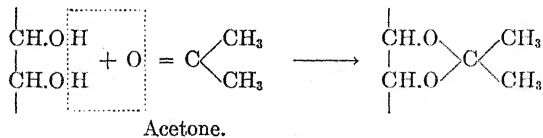
Formation of Ethers.—The ethers of sugars may be visualized as being formed by the condensation of the alcoholic hydroxyl groups of the sugar with the reacting alcohol.



Starting with the methylglycoside, the hexose derivatives have four free hydroxyl groups and therefore on complete methylation give the tetramethyl methylhexosides. Similarly, the pentose derivatives give the trimethyl methylpentosides.

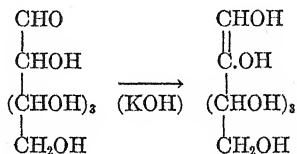
Whereas a glycosidic methoxyl group may be hydrolyzed with dilute mineral acid, those attached to the other hydroxyl groups are all true ethers and cannot usually be removed by any reagent except concentrated hydriodic acid (which gives methyl iodide and decomposes the sugar).

Formation of Cyclic Acetals.—The discovery of sugar acetals we owe to Fischer⁵ who, in 1895, showed that sugars condense with one or more molecules of acetone or benzaldehyde, with the elimination of water, giving isopropylidene and benzylidene compounds which are usually beautifully crystalline substances. v. Ekenstein later (1903) prepared certain methylene derivatives of sugars by condensation with formaldehyde.



The former are readily formed by shaking an acetone suspension of the finely divided sugar in the presence of a small amount of hydrogen chloride, zinc chloride or anhydrous copper sulfate. The acetone groups are readily hydrolyzed off by dilute mineral acids but not by alkali.

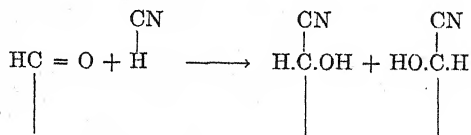
Aldehydic and Ketonic Properties.—All reducing sugars turn first yellow and then brown under the influence of alkali. It has been suggested that this is due to enolization, the wandering of a hydrogen atom:



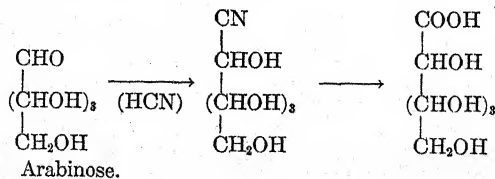
A double bond results and the consequent "oscillation" is said to cause color.

Both the aldoses and ketoses are readily oxidized and are consequently reducing agents. On warming with Fehling's solution (an alkaline copper solution), red cuprous oxide is precipitated—though the reaction is quantitative only if the conditions be accurately adjusted. Likewise, ammoniacal silver solutions are reduced to give a silver mirror.

Addition.—As shown by Kiliani⁶ the aldoses and ketoses combine directly with hydrogen cyanide, an addition compound (the cyanhydrin) resulting. Two stereo-isomeric forms are obviously capable of existence; they are usually formed simultaneously, one however predominating.

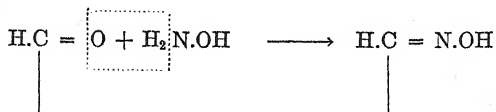


This reaction is very important as a means of "ascent" of the sugar series (*e. g.*, passing from a pentose to a hexose). Thus, Fischer performed the following synthesis:

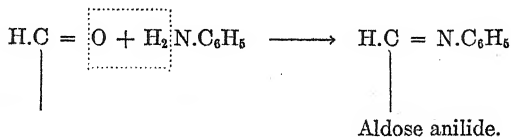


This mixture of gluconic and mannonic acids is separated through the phenylhydrazides and the two acids individually lactonized and then reduced to give glucose and mannose respectively.

Condensation.—The aldoses and ketoses condense with hydroxylamine to form the aldosozone (which is here represented in the non-cyclic form, though it may also exist in its cyclic forms):

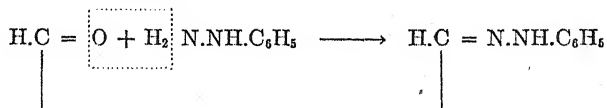


and with primary bases like aniline:



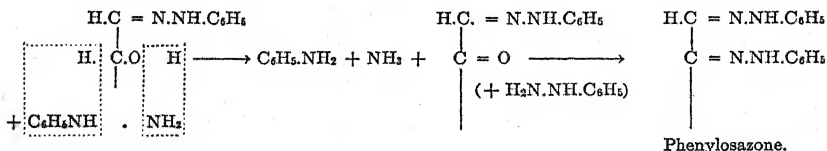
The anilides, which may have a heterocyclic structure, are beautifully crystalline substances, useful as a means of identification.

With phenylhydrazine the aldoses and ketoses condense in the cold, one molecule of the aldose or ketose reacting with *one* molecule of phenylhydrazine to give a phenylhydrazone:



These substances may exist in the cyclic forms, since they mutarotate readily when dissolved in aqueous alcohol or in pyridine.

However, on heating at 100° C. in the presence of excess phenylhydrazine, the reaction proceeds beyond the hydrazone and involves a total of *three* molecules of phenylhydrazine to give a phenylosazone. The reaction succeeds best at about pH 5, so a suitable proportion of acetic acid is added.



The osazone obtained is very distinct in its properties and characteristic for different sugars, and consequently affords a means of identification. Glucose, mannose and fructose all give the same osazone. This gave early investigators an insight into their structures. They obviously differ only in the groups attached to the first two carbon atoms. Now, glucose and mannose are aldoses, and fructose is a ketose. Hence their formulae must bear the following relationship:



Glucose.



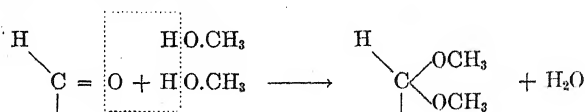
Mannose.



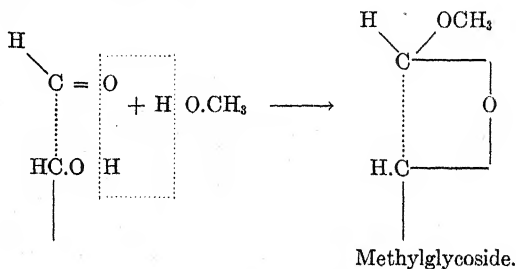
Fructose.

Modified phenylhydrazines will also give osazones. Thus, *p*-bromophenylhydrazine is often used. However, under the usual conditions, methylphenylhydrazine will speedily give an osazone with ketoses but much less readily with aldoses.

The completely methylated sugar 2. 3. 4. 5. 6-pentamethyl glucose condenses with *alcohols* to form the corresponding acetal, behaving just like any simple aldehyde.



When, however, the hydroxyl groups of the sugar are not protected, one of them enters into the reaction so that only *one* molecule of the alcohol condenses with the sugar. Cyclic inner half-acetals, known as *glycosides*, are thus produced.

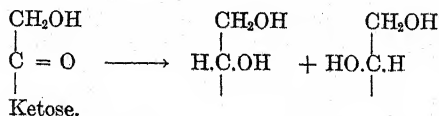


Under the catalytic action of dry hydrogen chloride all the free reducing sugars condense with dry methyl alcohol to form methylglycosides.

Reduction.—On reduction with sodium amalgam the monoses give the corresponding sugar alcohols, with the addition of two atoms of hydrogen, thus:

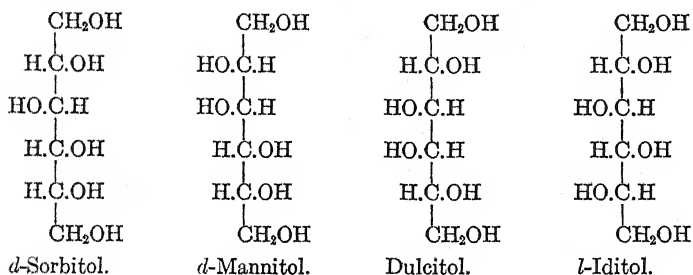


and



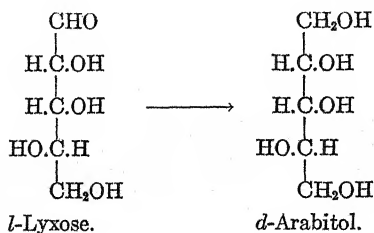
These alcohols are of interest since a number of them are widely distributed in plants. They are not fermentable but are attacked by a variety of molds and bacteria. Glycerol (the 3-carbon alcohol) occurs in nature as a component of fats. Erythritol (the 4-carbon alcohol) is also widely distributed in nature, the meso- or *cis*-form occurring as its esters in algae. Of the four possible pentahydric alcohols, only ribitol occurs in nature. Of the ten possible hexahydric alcohols, only four occur in nature, namely, *d*-mannitol, *d*-sorbitol, dulcitol and *l*-iditol. *d*-Glucose gives *d*-sorbitol; *d*-mannose gives *d*-mannitol, and *d*- or *l*-Galactose gives dulcitol.

Fructose yields both sorbitol and mannitol, since a new asymmetric center arises from the reduction of the ketonic group. Similarly, *d*-sorbitol and *l*-iditol are obtained from *l*-sorbosose.



Mannitol is very widely distributed—in manna, sap, leaves and fruits—and in certain fungi it exceeds glucose in quantity. *d*-Sorbitol is found in the ripe berries of the mountain ash, and in the fruits of most of the *Rosaceae*. It is formed by reduction of *l*-sorbosose (together with *l*-iditol), of *d*-fructose (together with *d*-mannitol), and of *d*-glucose. *l*-Iditol is also present in the berries of the mountain ash. Dulcitol occurs in "Madagascar" manna, which grows in Asia Minor.

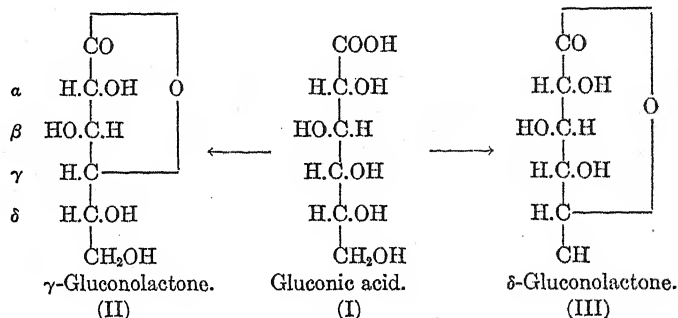
These substances are useful as foods, especially as bacteriological foods (culture media). The sugar alcohols have proved of value in establishing the configuration of the sugars, *e. g.*, *l*-lyxose may be reduced to *d*-arabitol—identical with the alcohol obtained by reducing *d*-arabinose.



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They are sweet, crystalline compounds which are soluble in water and alcohol. They give acetyl and benzoyl derivatives and condense with both acetone and benzaldehyde. The nitro compounds are explosive.

Oxidation.—When treated with bromine water, the ketoses remain unchanged whereas the aldoses undergo oxidation. Thus, glucose forms the monobasic sugar acid, gluconic acid (I), the “aldehydic” group being oxidized to carboxyl. On heating the acid for a short time at 100° C. to remove the elements of water, it very readily passes to its γ -lactone (II), a substance having m.p. 135° C.



On the other hand, in 1914 Nef⁷ described the isolation of a second lactone, having m.p. 152° C., from the oxidation product of glucose. Haworth and Nicholson⁸ showed this to be the δ -gluconolactone (III). It is distinguished from the γ -lactone by its ease of hydration to the parent gluconic acid. The δ -lactone is prepared by heating the free acid or its ester for a prolonged period below 100° C.

In aqueous solution an equilibrium is established between the monobasic acid and both its lactones. The rate of hydration of a lactone to the free acid can be measured polarimetrically since the optical rotation changes as the proportion of lactone diminishes. Alternatively the increase of conductivity of the aqueous solution caused by increase in free acid may be determined. The lowest rate observed for any of the δ -lactones is considerably greater than the highest rate of hydration of a γ -lactone.

The lactones of the unmethylated sugars may be transformed to the parent sugars by reduction with sodium amalgam in the presence of dilute sulfuric acid.

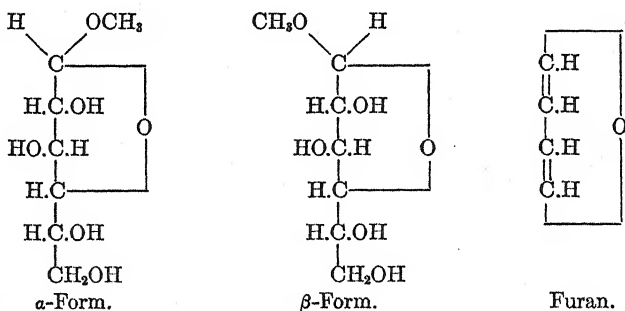
Glycosides.—The majority of sugars occur in nature in the form of glycosides. Their variety may be seen from a few typical examples shown in the table on page 53.

As previously mentioned, all the reducing sugars condense with dry methyl alcohol to form methylglycosides, under the catalytic influence of dry hydrogen chloride. Of the various ring-forms of the glycosides which might possibly be formed, only two types have actually been isolated.

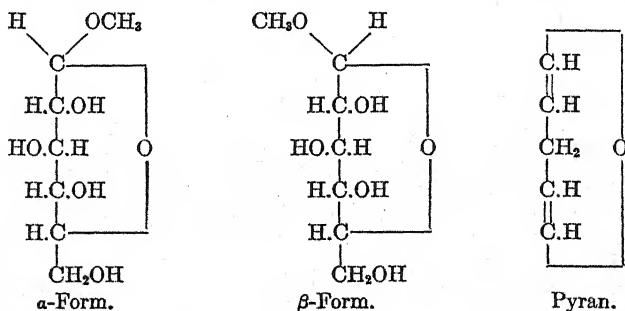
| Glycoside. | Products of hydrolysis. | |
|---------------------|-------------------------|-------------------|
| | Sugar. | Other product. |
| Arbutin..... | Glucose | Hydroquinone |
| Coniferin..... | Glucose | Coniferyl alcohol |
| Helicin..... | Glucose | Salicylaldehyde |
| Quercitrin..... | Rhamnose | Quercetin |
| Idain..... | Galactose | Cyanidin |
| Barbaloin..... | Arabinose | Aloemodin |
| Adenosine..... | Ribose | Adenine |
| Thymoguanosine..... | 2-Desoxyribose | Guanine |

Those derivatives in which the oxygen-ring bridges carbon atoms 1 and 5 in the aldoses (or carbon atoms 2 and 6 in the ketoses) have been termed *pyranose* by Haworth⁹ in order to indicate their relationship to pyran. Sugar derivatives in which the oxygen-bridge links carbon atoms 1 and 4 (or 2 and 5 in the ketoses) he has designated as *furanose* in order to emphasize their relationship to furan.

In the cold this reaction¹⁰ leads initially to the simultaneous formation of the α - and β -forms of the glycofuranoside. Thus glucose gives rise to a mixture of α - and β -methylglucofuranosides.



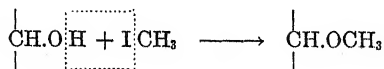
However, on long standing, or on heating at 66° C., the forms of this less stable type are transformed to the more stable pyranoside forms.



The glycosides are nonreducing toward Fehling's solution and have no aldehydic or ketonic properties. The furanosides are distinguished from the pyranosides by their ease of formation and their ready hydrolysis by extremely dilute mineral acid. In the case of the methylglucosides, for example, the furanoside is completely hydrolyzed by 0.01 *N* hydrochloric acid, whereas the pyranoside must be heated with *N* acid in order to effect hydrolysis. However, the nature of the aglucone has a powerful effect on the stability of the glycosides—the pyrimidine nucleosides, for example, being extremely resistant to hydrolysis despite their furanose structure. Glycosides are also hydrolyzed by enzymes, but the two isomers (α - and β -) are not hydrolyzed by the same enzyme. The action of enzymes is specific; thus, α -methylglucopyranoside is hydrolyzed by maltase, whereas β -methylglucopyranoside is hydrolyzed by emulsin. α -Methylglucofuranoside is not hydrolyzed by zymoin or emulsin.

Methylation. The Ring Structure of Glycosides.—The ring structures of the two types of glycoside (furanose and pyranose) have been established by a study of the products obtained by complete methylation.

There are two important methods for preparing ethers of sugars. The first was introduced by Purdie and Irvine¹² in 1903, and consists in heating the sugar derivative with the alkyl iodide in the presence of silver oxide (which removes the hydrogen iodide formed). Thus, methyl iodide gives the methyl ether:



This method can only be employed for glycosides and other derivatives in which the reducing group is either substituted or lacking. Attempts to methylate a free sugar by this method lead to oxidation by the silver oxide.

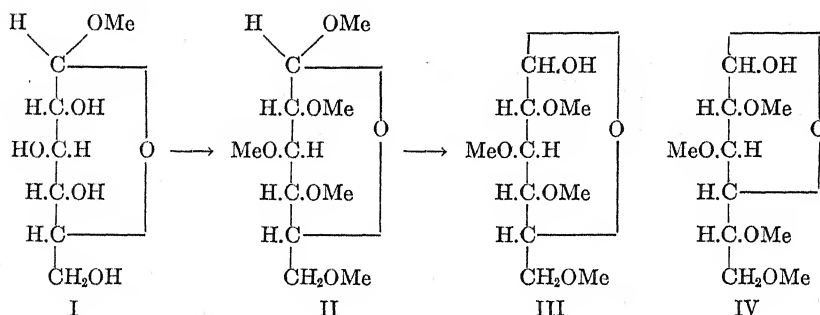
In 1915, Haworth¹³ first applied to the simple sugars and their derivatives a modification of the method which had been previously used without much success by Denham and Woodhouse for the methylation of cellulose. This consists in warming the aqueous solution during the slow simultaneous addition of equivalent proportions of sodium hydroxide solution and dimethyl sulfate.

In both cases the reaction, being heterogeneous, is facilitated by mechanical stirring, this consideration being important in Haworth's method in order to prevent even transitory local acidity which might cause hydrolysis, or excessive alkalinity which would induce enolization; and in Purdie's method to prevent oxidation and to accomplish methylation in one treatment.¹⁴

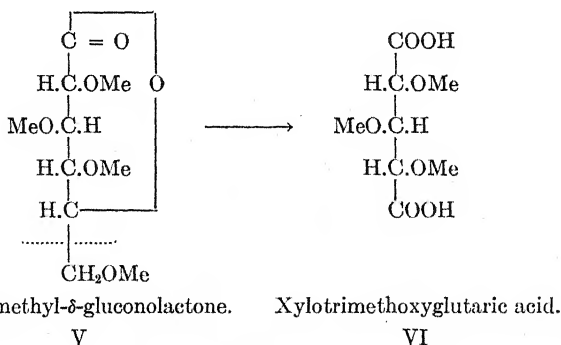
Haworth's method is preferable for a number of reasons. It may be used for the direct methylation of reducing sugars, by employing a

slight excess of dimethyl sulfate until all reducing action has disappeared and then maintaining a slight alkalinity during the remainder of the treatment. Secondly, it may be used for methylating substances insoluble in methyl iodide. There is no danger of oxidation occurring and the methylating agents employed are cheap and readily accessible.

Methylation of α -methylglucoside (I) by either method gives tetramethyl- α -methylglucoside (II), from which tetramethyl glucopyranose (III) is obtained on hydrolysis. In a similar way α -methylglucofuranose, by methylation and subsequent hydrolysis, yields tetramethyl glucofuranose (IV).



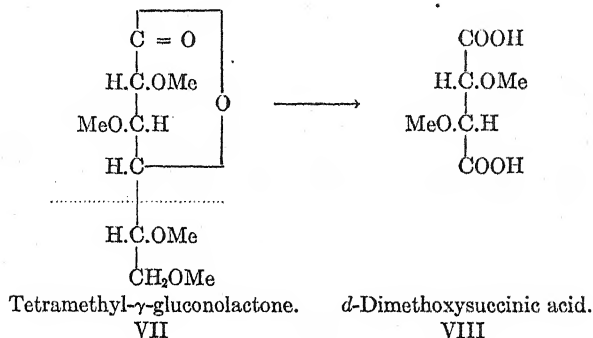
The tetramethyl glucose (III) from either α - or β -methylglucoside is readily oxidized by bromine water to the tetramethyl gluconic acid, which lactonizes to give tetramethyl- δ -gluconolactone (V). That it is the δ -lactone is shown by its rapid rate of hydration to the acid and by further oxidation with concentrated nitric acid to give xylotrimethoxyglutaric acid (VI).¹⁵ The same final product is obtained by direct oxidation of the methylated sugar with nitric acid.



It is therefore quite evident that the tetramethyl glucose had the pyranose ring structure, and this formulation is extended to the α - and β -methylglucosides from which it was prepared. In the same way the

normal forms of the glycosides of galactose, mannose, fructose, arabinose, xylose, ribose, and lyxose have been shown to possess the pyranoside structure.

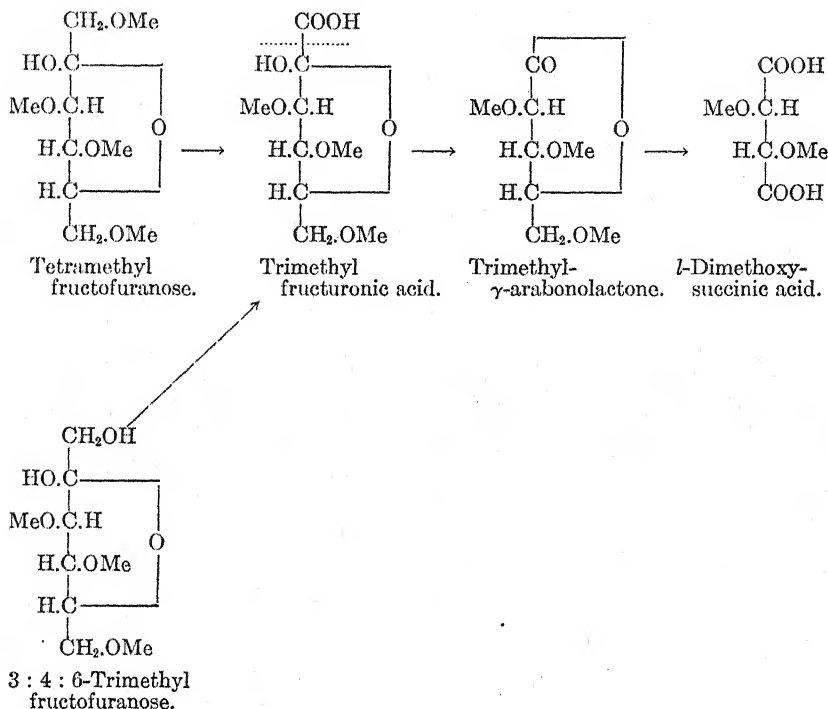
Turning now to tetramethyl glucofuranose (IV), it is found that bromine oxidation and subsequent lactonization lead to the formation of tetramethyl- γ -gluconolactone (VII). Its hydration curve clearly differentiates it from the tetramethyl- δ -gluconolactone, since it is hydrated extremely slowly. On further oxidation it gives *d*-dimethoxy succinic acid (VIII).¹⁵



It follows that the tetramethyl glucose from which the lactone was derived must be formulated as 2:3:5:6-tetramethyl glucose and that the parent glycoside was a furanoside.

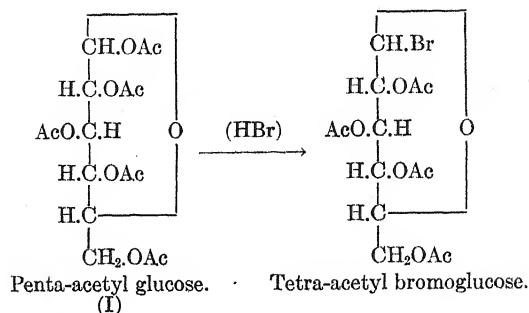
In the same way it has been demonstrated that the glycosides formed at room temperature from galactose, mannose, fructose, arabinose, xylose, and lyxose possess the furanoside structure. Ribose is widely distributed in nature in the furanose form, since it is a component¹⁶ of adenosine, guanosine, cytidine, and uridine, all of which are constituents of nucleic acid. Combined fructose occurs in nature only in the furanose form. It is a component of sucrose, raffinose, gentianose, inulin, and levan.

The tetramethyl fructose obtained by hydrolysis of methylated sucrose and the trimethyl fructose from methylated inulin were shown to have the furanose structure in the following way: On oxidation with nitric acid the terminal group (on carbon atom 1) was transformed to a carboxyl group, yielding a trimethyl fructuronic acid which, on further oxidation (with potassium permanganate and dilute sulfuric acid), was converted to trimethyl γ -arabonolactone. When subjected to degradative oxidation with nitric acid, *l*-dimethoxysuccinic acid was formed:

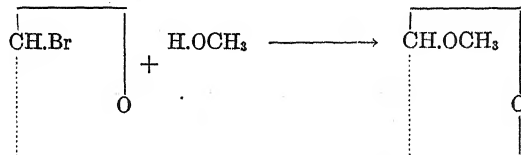


The Sugar Poly-esters.—The sugar esters are derivatives which have played an important part in the development of our knowledge of the sugars. In the first place they furnished evidence as to the number of free hydroxyl groups in the monosaccharide molecule. If an excess of the esterifying agent is employed, to insure complete esterification, it is found that the hexoses give the penta-ester [*e. g.*, glucose pentacetate (I)] and the pentoses give the tetra-ester (*e. g.*, xylose tetra-acetate). In general, for a sugar having the empirical formula $\text{C}_n\text{H}_n(\text{OH})_n$, only $(n-1)$ hydroxyl groups are capable of esterification. This discovery was one of the factors leading to the adoption of the cyclic formulation for the sugars, a formulation which required that two forms (α - and β -) of each sugar acetate should exist. Two such stereo-isomeric forms of acetate have now been isolated for all the common sugars.

The sugar acetates are of particular interest for another reason. In both isomerides (α - and β -) of the fully acetylated monoses the acetyl group attached to the carbon atom which originally carried the "reducing" group is far more active than the others. When subjected for one hour to the action of a solution of dry hydrogen halide in glacial acetic acid, this acetyl group alone is replaced by halogen, thus:



The bromoacetyl derivatives have proved to be of immense value for the synthesis of glycosides (including di- and polysaccharides). The halogen atom is even more reactive than the acetyl group which it replaced, and on shaking with silver carbonate and an alcohol the compound gives the tetracetyl glycoside (when the sugar has *trans* groupings at positions 2 and 3):

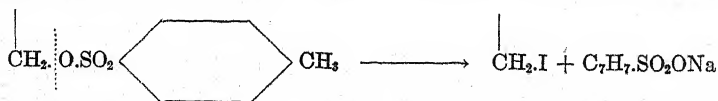


The bromoacetates also condense with phenols in the presence of alkali; with morphine; with potassium sulfide to give sulfur derivatives; with the silver salts of thiourethanes to give mustard oil glucosides and with the silver compounds of some of the purines, giving glycosides termed *nucleosides*.

These bromoacetyl derivatives also serve as the starting material for the preparation of glycosens, glycals and desoxysugars.

The sugar acetates and benzoates are colorless, crystalline compounds, practically insoluble in cold water and readily hydrolyzed to the parent sugar by means of dilute alkali. Vaciniin or monobenzoyle glucose occurs in the whortleberry, and the tannins are probably gallic acid esters of glucose.

The *p*-toluene-sulfonyl esters have recently proved of use in ascertaining the structure of sugar derivatives. It has been found that a *p*-toluene-sulfonyl group attached to position 5 in a pentofuranose or position 6 in a hexopyranose (*i. e.*, the primary hydroxyl group) is readily replaced by iodine on treatment with sodium iodide in acetone:



On the other hand, when substituted in other positions, this substituent is much more tenaciously bound.

THE ACETATES AND 1-HALOGEN ACETATES OF THE ALDOPENTOSE AND ALDO-HEXOSES.

| Substance. | | M. P. (degrees). | Specific rotation (degrees). |
|----------------------|--------------------------|---------------------|---------------------------------|
| α -Tetracetyl | <i>l</i> -arabinose..... | 97 | + 42.5 (in chloroform) |
| β - " | <i>l</i> -arabinose..... | 86 | +147.2 " " |
| α - " | <i>d</i> -xylose..... | 59 | + 88.9 " " |
| β - " | <i>d</i> -xylose..... | 128 | - 25.1 " " |
| α - " | <i>d</i> -lyxose..... | 93-94 | + 25 " " |
| β - " | <i>d</i> -ribose..... | 110 | - 52 " " |
| α -Pentacetyl | <i>d</i> -mannose..... | 75 | + 57.6 " " |
| β - " | <i>d</i> -mannose..... | 117-118 | - 25.3 " " |
| α - " | <i>d</i> -galactose..... | 95.5 | +106.7 " " |
| β - " | <i>d</i> -galactose..... | 142 | + 25.0 " " |
| α - " | <i>d</i> -glucose..... | 114-114.5 | +101.6 " " |
| β - " | <i>d</i> -glucose..... | 135.5 | + 3.8 " " |
| Bromotriacetyl | <i>l</i> -arabinose..... | 139 | +287.1 " " |
| " | <i>d</i> -xylose..... | 102 | +212.2 " " |
| " | <i>d</i> -ribose..... | 96 | -209.3 " " |
| Bromotetracetyl | <i>d</i> -mannose..... | 53-54 | +123.2 " " |
| " | <i>d</i> -galactose..... | 85 | +236.4 (in benzene) |
| " | <i>d</i> -glucose..... | 88-89 | +197.8 (in chloroform) |

Partially Substituted Sugars.—The acetone and benzylidene derivatives are of particular significance since they have been employed for the preparation of partially substituted sugars, the substituents occupying definitely known positions. The preparation of such derivatives was desirable, as partially esterified sugars occur in nature and partially methylated sugars are obtained by the hydrolysis of methylated di-, tri-, and polysaccharides. Being acetals, these acetone and benzylidene derivatives are stable toward alkali, but readily hydrolyzed by acids.

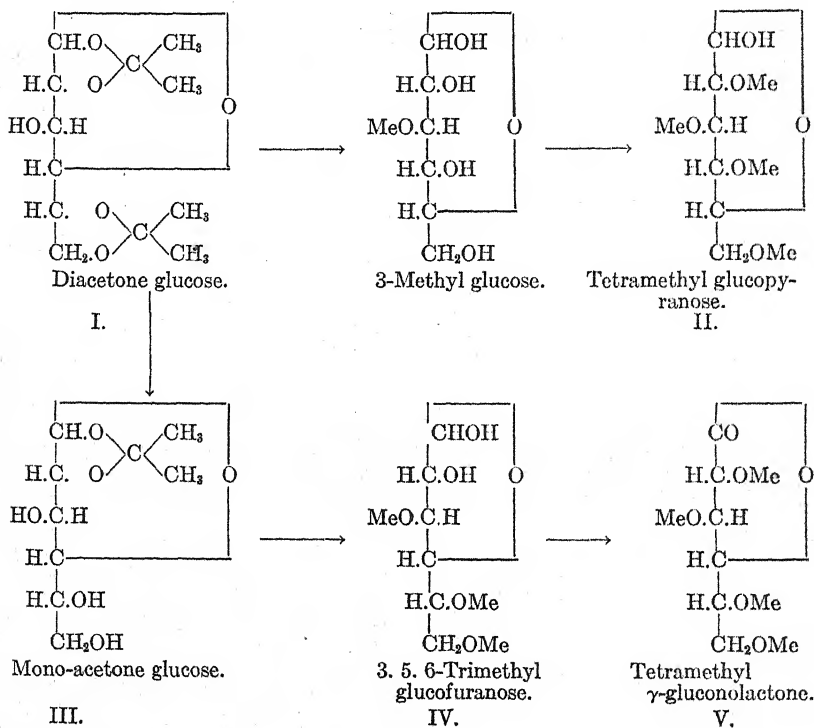
The methylation method has proved of value in elucidating the structure of the acetone sugars and has, incidentally, rendered available a number of partially substituted sugar derivatives.

Glucose forms a beautifully crystalline diacetone compound (di-iso-propylidene glucose (I), p. 60). Four of the hydroxyl groups in the original sugar have been protected by the two acetone residues and consequently on methylation a monomethyl diacetone glucose is formed. On hydrolysis the acetone residues are eliminated and a crystalline monomethyl glucose results.

Since on complete methylation and hydrolysis this is transformed into crystalline 2:3:4:6-tetramethyl glucopyranose (II), it follows that the methoxyl group in the original monomethyl glucose was situated at one of these four positions and its presence at position 5 is excluded. On oxidation¹⁷ it yields a monomethyl saccharolactone which, on reduction, gives monomethyl-*d*-glucuronic acid. From these results it is inferred that the methoxyl group is neither in the 6-position, since

this position is oxidized to a carboxyl group without the loss of a methoxyl, nor is it in position 4, since this position is occupied by the lactone ring in the monomethyl-*d*-saccharolactone.

Now, on careful hydrolysis of glucose diacetone, a glucose monoacetone (III) which is nonreducing toward Fehling's solution is obtained. On methylation and hydrolysis this gives a trimethyl glucose (IV) which forms a crystalline phenylosazone. It follows that the monoacetone derivative was 1:2-isopropylidene glucose (III). Since the trimethyl glucose is readily transformed by methylation and oxidation to tetramethyl γ -gluconolactone (V), the trimethyl glucose is a glucofuranose, and glucose diacetone is 1:2, 5:6-di-isopropylidene glucofuranose.



In a similar way the structure of other acetone derivatives of sugars has been elucidated. The diacetone derivatives of mannose and xylose are likewise furanose, whereas those of galactose and fructose are pyranose. Ribose forms a furanose monoacetone derivative, but the diacetone derivative has not, as yet, been prepared.

It is seen that acetone residues condense with *cis* hydroxyl groups on neighboring carbon atoms in any sugar, and the oxide ring of the sugar will shift in order to accommodate the preferential selection of positions of entry for the acetone residues.

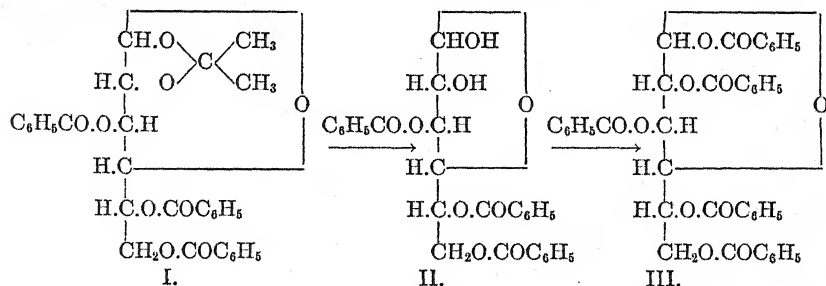
It has been shown that the acetone residues in diacetone glucose are situated on carbon atoms 1: 2 and 5: 6. Consequently diacetone glucose may be methylated or phosphorylated to give the 3-substituted derivative. The acetone groups may then be hydrolyzed off, yielding 3-methyl- or 3-phosphoglucose.

Furthermore, an acetone or benzylidene group attached at the 1: 2 positions is somewhat more stable than one attached at other positions. It is thus possible to hydrolyze only one acetone group from diacetone glucose (I), for example, to give the monoacetone derivative (III).

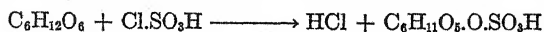
Two forms of the benzylidene derivative of monoacetone glucose have been prepared, which differ in the position of the free hydroxyl group. 1: 2-Monoacetone-5: 6-benzylidene glucose is formed by the action of benzaldehyde on monoacetone glucose in the presence of anhydrous sodium sulfate during five hours at 140°–150° C. Like diacetone glucose, it may be employed for the synthesis of 3-substituted derivatives.

If the reaction is arrested after thirty minutes, however, the product is 1: 2-monoacetone-3: 5-benzylidene glucose. It may be used for the preparation of 6-substituted derivatives of glucose.

The acetone and benzylidene derivatives are of special value since, in the case of certain sugars, they are of the furanose form and so afford a readily accessible material for the preparation of substituted furanose derivatives. Thus, complete benzoylation of monoacetone glucose gives tribenzoyl monoacetone glucofuranose (I). The acetone group may be hydrolyzed off, without removing the benzoyl groups, to give 3,5,6-tribenzoyl glucofuranose (II), a substance which is of interest since it may be further benzoylated to give the crystalline α - and β -pentabenzoyl derivatives (III) of glucofuranose.



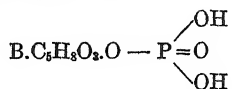
When only one molecular proportion of the esterifying agent is employed, the tendency is for the primary hydroxyl group to be preferentially esterified. Thus, glucose 6-monosulfonic acid (which occurs in nature in agar) may be prepared by treating glucose with chlor-sulfonic acid:



Similarly, the cautious phosphorylation of monoacetone glucose gives monoacetone glucose 6-phosphate, from which the acetone residue is

readily hydrolyzed to give 6-phosphoglucose (Robison's ester). 5-Phosphoribose and 5-phosphouridine have been synthesized in a somewhat similar manner.

The sugar phosphates play an important part in fermentation and in the structure of the nucleic acids. Yeast nucleic acid is composed of four mononucleotides, each of which is a glycoside of ribose phosphoric acid having the general formula



where B represents the bases adenine, guanine, cytosine and uracil.

The hydrolysis of sugar phosphates has been studied by Levene and coworkers. The position of the phosphoryl radicle profoundly affects their stability. Thus, 3-phosphoribose is much more readily hydrolyzed by dilute mineral acid than is 5-phosphoribose.

The trityl ethers of sugar derivatives show the same tendency to form on the terminal atom owing to the greater reactivity of the primary alcoholic group. They may be prepared by the action of triphenylmethyl chloride in pyridine solution and, unlike the methyl and ethyl ethers of sugars, are readily hydrolyzed by halogen acid in glacial acetic acid or in methyl alcohol. They have served for the synthesis of a variety of partially substituted sugar derivatives and of disaccharides (*e. g.*, gentiobiose).

By reactions of these types, four of the five monomethyl glucoses have now been synthesized:

| | | |
|---|----------------|---|
| 2 | Methyl glucose | (from 3,4,6-triacetyl glucose). |
| 3 | " " | (" diacetone glucose). |
| 4 | " " | (" 2,3,6-tribenzoyl- β -methylglucoside). |
| 6 | " " | (" 2,3,4-tribenzoyl- α -methylglucoside). |

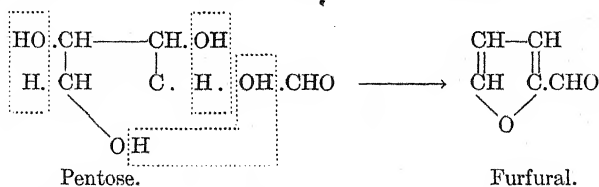
They are of interest since the 4-methyl glucose should be incapable of forming a furanose form of methylglycoside and the 5-methyl glucose should not give a pyranose glycoside, whereas the three other methyl glucoses should give glycosides of both types. These conclusions have been verified in the cases of 4-methyl- α -glucoheptose and 5-methyl ribose.

Of the four trimethyl glucoses, two (2,3,6-trimethyl glucose and 2,3,4-trimethyl glucose) are important as they are found in the hydrolytic products of methylated di-, tri- and polysaccharides. 2,3,4-Trimethyl glucose may be prepared by methylation and hydrolysis of 6-trityl- α -methylglucoside and is obtained by hydrolytic cleavage of methylated gentiobiose. 2,3,6-Trimethyl glucose is obtained by methylating and then hydrolyzing cellulose, cellobiose, starch, and glycogen.

Naturally Occurring Sugars and Sugar Derivatives.—*Pentoses* ($\text{C}_5\text{H}_{10}\text{O}_5$).—Of the eight possible aldopentoses, four occur in nature. The sugars *l*-arabinose and *d*-xylose are usually found as polysaccharides of high molecular weight, known as *pentosans*. They also occur

as glycosides, but have not been found as the free sugar. Xylose and arabinose serve as nutrient to yeast and bacteria, but plants have apparently no power of utilizing them. The pentosans are hydrolyzed by dilute mineral acid, but not by alkali. Thus, xylan may be extracted from esparto cellulose by boiling with sodium hydroxide solution. On pouring the extract into alcohol the xylan is precipitated.

The pentoses are readily distinguished from the other monosaccharides owing to their ready conversion to furfural on boiling with concentrated hydrochloric acid. The amount of furfural formed may be determined quantitatively by coupling with phloroglucinol and weighing the insoluble product. In this way the amount of pentose in a mixture of pentoses and hexoses may be determined.



A useful qualitative test for a pentose is with a reagent introduced by Tollens—a solution of orcinol in 18 per cent hydrochloric acid, which gives a deep purple color on boiling with a pentose. In the presence of a little ferric chloride a *green* color is obtained.

Hexoses, $\text{C}_6\text{H}_{12}\text{O}_6$.—Of the 16 possible aldohexoses, but four optically active forms are known to occur in nature. The other 12 aldohexoses have been synthesized successfully, but only glucose, galactose, mannose and the ketose, fructose, are of physiologic interest. Free fructose occurs in nearly all sweet fruits and in honey. In combination, it is widely distributed in nature.

Glucose is the most important of the sugars, being the form in which carbohydrate is carried in the blood and is oxidized by the tissues.

The hexoses differ from most other true sugars in two important respects. On boiling with dilute mineral acids, levulinic acid (which may be identified as its phenylhydrazone) is formed and, excepting glycerose and mannnonose, the naturally occurring hexoses (*d*-glucose, *d*-mannose, *d*-galactose and *d*-fructose) are the only sugars which undergo fermentation by yeast. Hexoses give a red color on warming with resorcinol in hydrochloric acid; this coloration forms more rapidly with ketohexoses than with aldohexoses.

The hexoses may also be distinguished by Fenton's test. A small sample is moistened with water and warmed with a little phosphorus tribromide until the mixture darkens. It is then cooled and mixed with a little alcohol and ethyl malonate. On adding an excess of alcoholic potash and diluting with water, a blue fluorescence appears.

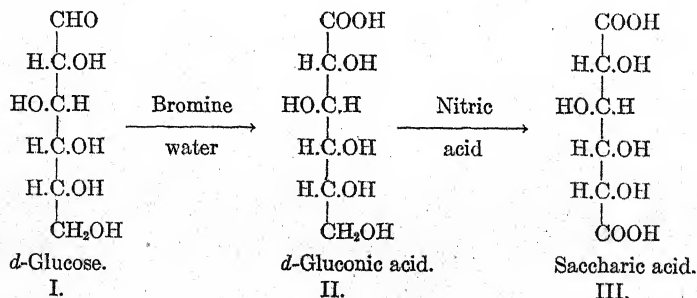
Aldohexoses may be distinguished from one another by the nature of the dibasic sugar acid formed on oxidation with nitric acid, by their phenylhydrazones and phenylosazones.

| Sugar. | Source. |
|---|--|
| (a) <i>Aldopentose</i> <i>d</i> -ribose..... | Sugar component of yeast nucleic acid. |
| <i>d</i> -arabinose.. | Sugar component of the glycoside aloin; in tubercle bacilli. |
| <i>l</i> -arabinose | As the polymerized anhydride (araban) in rye and wheat bran, seed shells and fruit skins; various gums such as gum arabic and cherry gum; beet pulp; also combined with glucose as the disaccharide <i>vicianose</i> . |
| <i>d</i> -xylose.... | As the polymerized anhydride (xylan) in wood gum, straw, corn cobs, cotton-seed hulls, bamboo. |
| (b) <i>Aldohexose</i> <i>d</i> -glucose.... | As the free sugar; as polymerized anhydride (starch, cellulose, glycogen); as glucosides and as a component of many di-, tri-, and tetrasaccharides; sucrose. |
| <i>d</i> , <i>l</i> -Glucose.. | In the <i>d</i> -, <i>l</i> -glucoside capsularin. |
| <i>d</i> -Galactose.. | As polymerized anhydride (galactan); as component of cerebro-sine; as glycosides and as component of many di-, tri-, and tetrasaccharides; lactose. |
| <i>l</i> -Galactose.. | In flaxseed mucilage. |
| <i>d</i> -Mannose.. | As glycosides; in glycoproteins; as polymerized anhydrides (mannans) and as component of di-, tri-, and tetrasaccharides; ivory nuts. |

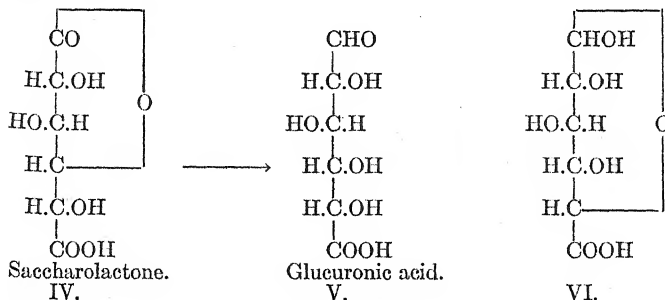
Glucuronic Acid.—Glucuronic acid is physiologically one of the most interesting oxidation products of glucose, being frequently found in the urine combined with a variety of substances in glycosidic union.

On oxidizing glucose (I) (or gluconic acid (II)) with nitric acid, besides the aldehydic group, the reactive primary hydroxyl group is attacked, giving the dibasic acid, saccharic acid (III). (In a similar way, galactose gives mucic acid.)

The free acid is crystalline but it readily passes to the lactone (IV), an equilibrium being set up in aqueous solution. The pure lactone is prepared by heating saccharic acid.



When saccharolactone is reduced with sodium amalgam and dilute sulfuric acid, it is converted into glucuronic acid (V). This substance is remarkable since it is both an aldehyde and an acid.

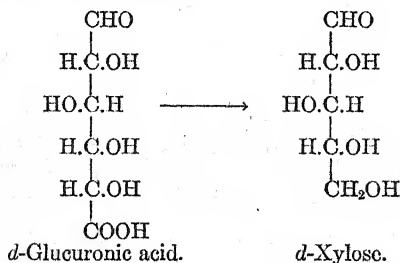


The animal organism normally oxidizes glucose rapidly to give carbon dioxide and water. When, however, substances (like chloral and camphor) which are oxidized with difficulty in the body are introduced, they combine with glucose to form glucosides. Oxidation then occurs at position 6 to give the glucuronic acid derivative which is excreted in the urine. This is one of the methods by which the body exerts a protective or detoxicating effect.

Glucuronic acid was formerly obtained from hydrolysis of euxanthic acid, a substance obtained in India from the urine of cows fed with mango leaves. It is no longer available since aniline dyes have displaced euxanthic acid from the market. Glucuronic acid is now obtained from the urine of rabbits or dogs after administering menthol. Pryde and Williams have isolated glucuronic acid from human and canine urine after administration of borneol, and have subjected it to methylation and subsequent hydrolysis. From the nature of the oxidation products they conclude that glucuronic acid has the pyranose ring (VI).

Glucuronic acid also occurs combined in plant materials (*e. g.*, gum arabic, the aldobionic acid from which is glucurono-6- α -galactose) and in specific polysaccharides, *e. g.*, that from *Friedländer bacillus* Type A gives glucurono-glucosé. The lactone of glucuronic acid occurs in nature in the saponins of the sugar beet.

By the action of certain bacteria (such as occur in decomposing flesh) it is converted into *d*-xylose.



The carbohydrate groups present in the mucins and other glucoproteins have the structure of polysaccharides in which glucosamine and glucuronic acid are united. It has been shown by Levene and La Forge,¹⁸ however, that the substances also contain sulfur, in the form of sulfuric acid ester-linkage with the sugars, while the amino group of the glucosamine is not free, but is acetylated. The carbohydrate group of all mucoproteins is conjugated with sulfuric acid. The group is thus built up of four components in equimolecular proportions: Sulfuric acid, acetic acid, hexosamine, and glucuronic acid.

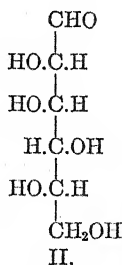
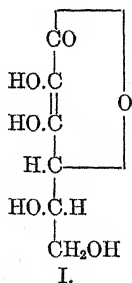
d-Galacturonic acid occurs in nature in lemon pulp. Pectin substances obtained from a large number of plants and vegetables are derivatives of galacturonic acid. It is obtained from the pectins of the sugar beet by heating with 1 per cent oxalic acid. It reduces cold Fehling's solution, gives furfural on heating with hydrochloric acid and is oxidized to mucic acid.

d-Mannuronic acid is obtained by the hydrolysis of alginic acid (from *macrocytis pyrifera*).

THE CONSTITUTION AND SYNTHESIS OF ASCORBIC ACID (VITAMIN C)

The investigation of oxidation systems of biological interest led to the discovery by Szent-Györgi⁵² of a crystalline substance, $C_6H_8O_6$, which possessed strong antiscorbutic properties. This substance had an acidic character, strong reducing power and color reactions similar to those given by carbohydrates and is considered by many workers to be vitamin C in pure crystalline condition. It may be isolated both from animal (*e. g.*, adrenal glands) and plant (*e. g.*, paprika) sources, and has been named *ascorbic acid*.

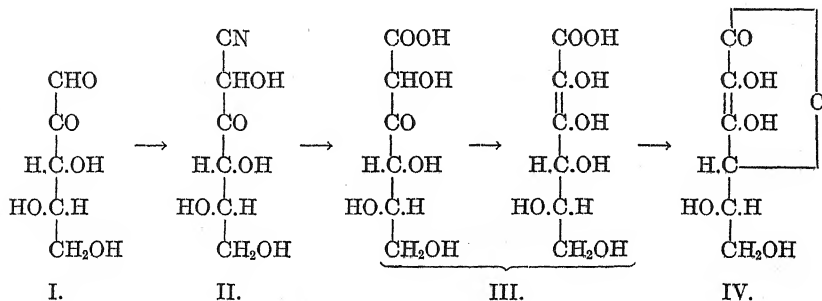
By studying its oxidation products, it was demonstrated that ascorbic acid (I) is a derivative of *l*-gulose (II), namely 3 keto-*l*-gulonolactone.



Furthermore, when tetramethyl ascorbic acid was oxidized by ozone, two atoms of oxygen were taken up to give a neutral product which, upon treatment with alcoholic ammonia, yielded the amide of oxalic acid and dimethyl-*l*-threonamide.⁵³

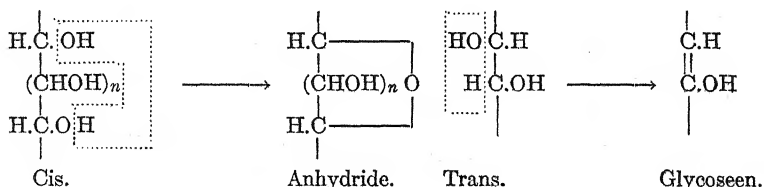
l-Ascorbic acid has been synthesized⁵⁴ from *l*-xylosone (I). On treatment with potassium cyanide and calcium chloride, the β -keto-

nitrile (II) was formed. After a few minutes this hydrolyzes to the β -keto acid, with elimination of ammonia. This intermediate product (III) is known as *pseudo-ascorbic acid*. It is quantitatively converted into ascorbic acid (IV) by means of 8 per cent aqueous hydrochloric acid at 40–50° C.



Anhydrosugars.—The anhydrosugars are of interest since they have occupied an important place in recent discussions of the structure of the polysaccharides. They are substances having the general formula $C_n(H_2O)_{n-1}$, and may be visualized as being formed from sugars by the elimination of the elements of water from two hydroxyl groups of a single monosaccharide molecule.

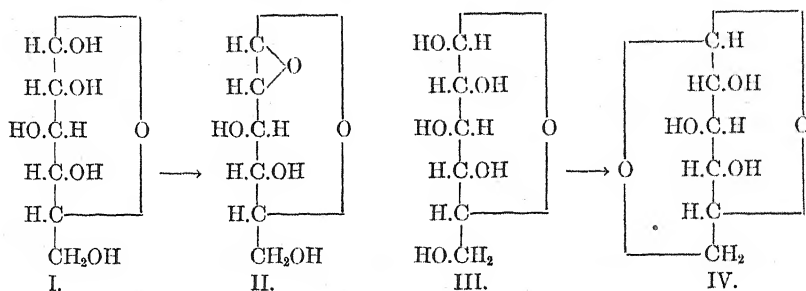
Substances of two types may be formed by this process. In the first, an oxygen bridge is established between two carbon atoms carrying the reacting hydroxyl groups in the *cis* position; in the second group a double bond forms between two adjacent carbon atoms having *trans* hydroxyl groups.



Substances of the first group are known as *sugar anhydrides* and the number of such derivatives which might conceivably be prepared from any one sugar would depend on the number of pairs of carbon atoms having hydroxyl groups in the *cis* position.

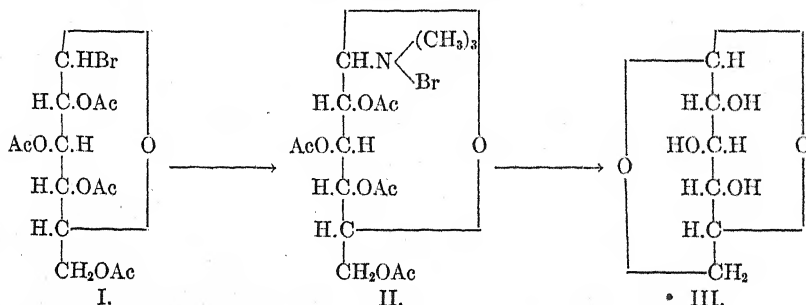
Substances of the second group are termed *glycoseens* and their number derivable from any one sugar would depend on the number of pairs of adjacent carbon atoms having *trans* hydroxyl groups.

Sugar Anhydrides.—By heating, α -*D*-glucose (I) under diminished pressure at 145° C. during two hours, α -*D*-glucosan (II), $C_6H_{10}O_5$, is said to be formed. Similarly, it is claimed that β -glucose (III) gives rise to β -glucosan (IV).



Other sugars yield similar products, the exact constitutions of which are as yet undecided.

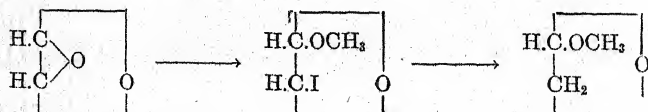
Karrer has prepared β -glucosan (1: 6-anhydroglucose) in the following manner: By treating bromotetracetyl glucose (I) with trimethylamine, tetracetylglucosido-trimethylammonium bromide (II) was obtained. On hydrolysis this gave β -glucosan (III).



The properties of the sugar anhydrides depend on the position of the new ring which has been acquired. Those in which it is formed between the "*reducing*" carbon atom and some other carbon atom of the chain are very reactive, a reactivity due to their having the structure and properties of acetals. This second ring, like the ethylene oxide ring, is readily severed by inorganic or organic acids to give esters and ethers. They can therefore be employed for the synthesis of disaccharides.

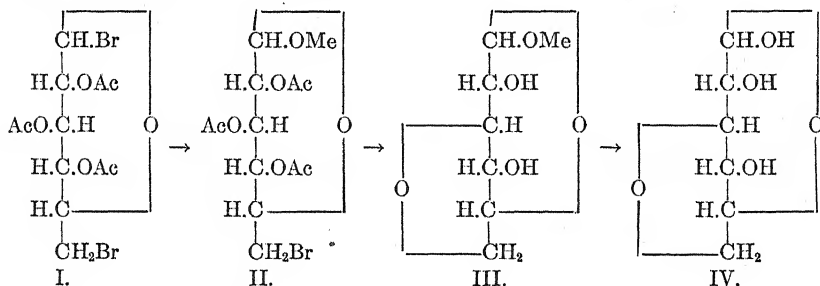
Thus, by the action of cold concentrated hydrochloric acid, β -glucosan gives α -chloroglucose, $\text{C}_6\text{H}_{11}\text{O}_5\text{Cl}$, which reacts with potassium glucosan to give a new α -glucosido-glucose resembling gentiobiose.

By treating α -glucosan with one molecular proportion of methyl iodide, methylglucoside 2-iodohydrin is formed. This is readily reduced to 2-desoxymethylglucoside.



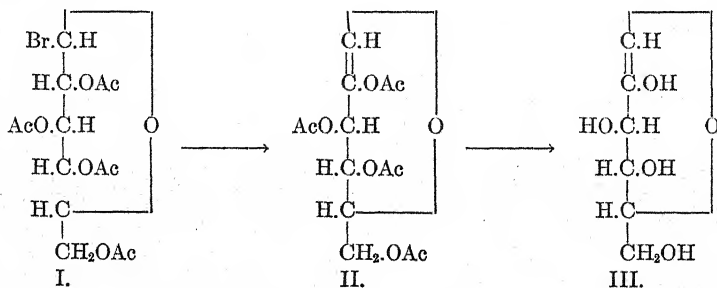
On the other hand, those sugar anhydrides in which the second oxygen bridge forms between *two alcoholic hydroxyl groups* are characterized by a much greater stability of the second ring. The first substance of this type to be prepared was *chitose*, obtained by the deaminization of glucosamin with nitrous acid. This sugar anhydride, like the products similarly obtained from the other 2-aminosugars, has the new oxygen ring between carbon atoms 2 and 5.

Fischer and Zach later prepared a sugar anhydride in which the new ring was said to be between carbon atoms 3 and 6. 1:6-Dibromotriacetyl glucose (I) was treated with methyl alcohol and silver carbonate to give 6-bromotriacetyl methylglucoside (II). This was deacetylated and the elements of acetyl bromide simultaneously removed by the action of barium hydroxide, and the resulting anhydromethylglucoside (III) was transformed into 3:6 (?) anhydroglucose (IV) by mild treatment with hydrochloric acid:



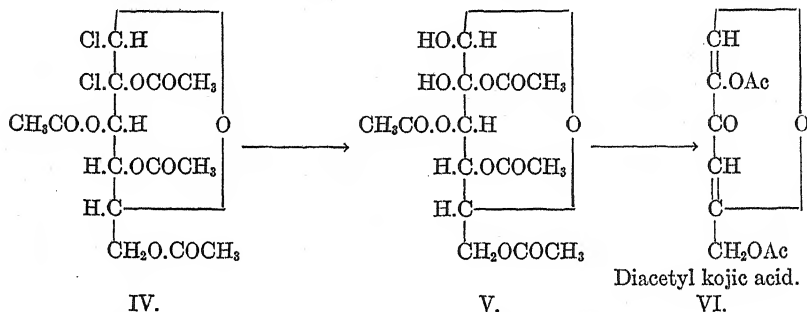
Glycoseens.—The glycoseens so far prepared are of two kinds, in which the double bond is between the first and second carbon atoms or the terminal and penultimate carbon atoms respectively.

The substances of the glycoseen (1,2) type, which may be regarded as 2-hydroxyglycals, are readily prepared from the bromoacetyl derivative and are of interest, being quite intimately related to substances of the type of ascorbic acid (vitamin C). Thus, the elements of hydrogen bromide may be eliminated from bromotetracetyl glucose (I), by warming with diethylamine, to give the unsaturated glucose derivative, tetracetyl glucoseen-1,2 (II). On deacetylation, glucoseen-1,2 (III) is obtained.



The importance of this substance lies in the fact that it is readily converted into kojic acid, a phenolic alcohol of pyrone, which is formed in nature by the growth of *aspergillus oryzae* on koji or steamed rice, or on glucose.

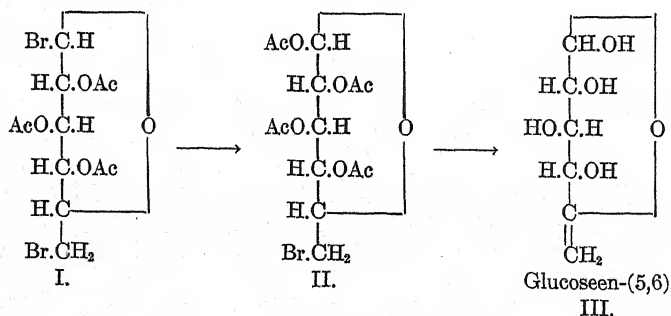
By the action of chlorine in anhydrous ether on tetracetyl glycoseen-(1,2), an unstable dichloride (IV), is formed. This is transformed by treating with silver carbonate and aqueous ether to 2,3,4,6-tetracetyl glucosone hydrate (V) (a substance which, when deacetylated and condensed with phenyl hydrazine, gives glucosazone). If treated with acetic anhydride and pyridine, diacetyl kojic acid (VI) is formed.



Galactoseen-(1,2) naturally gives the same product, since the asymmetry of the carbon atoms has been lost.

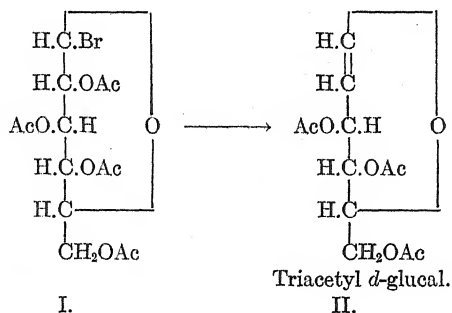
These derivatives may be used for the descent of the series. Thus, on oxidation with aqueous alkaline potassium permanganate solution, galactoseen-(1,2) is transformed to potassium lyxonate, from which lyxonolactone is readily obtained.

Glucoseen-(5,6).—By long treatment of pentacetyl glucose with liquid hydrogen bromide in a sealed tube not only the first but also the terminal acetyl group is replaced giving triacetyl 1,6-dibromoglucose (I). This is transformed, by heating with acetic acid and silver acetate, into β -tetracetyl glucose 6-bromohydrin (II) which gives the corresponding 6-iodohydrin by the action of sodium iodide in acetone at 100° C. Treatment of this with silver fluoride in pyridine, followed by deacetylation gives glucoseen-(5,6) (III).



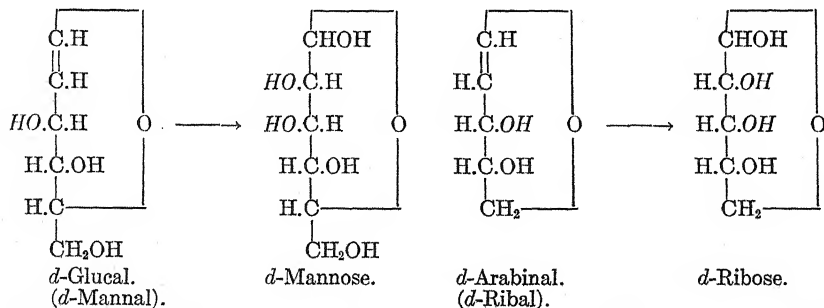
Glycals.—Another class of extremely important unsaturated sugar derivatives, in which the double bond may be visualized as forming by the elimination of *the elements of hydrogen peroxide* from the hydroxyl groups of adjacent carbon atoms, are known as the *glycals*. These derivatives have proved of use for the descent of the series, for the preparation of the epimers of sugars and in the synthesis of desoxy sugars.

By reduction of tetracetyl bromoglucose (I) with zinc dust in the presence of aqueous acetic acid, the elements of acetyl bromide are eliminated, giving glucal triacetate (II) the pyranose ring structure of which has been revealed by methylation studies.



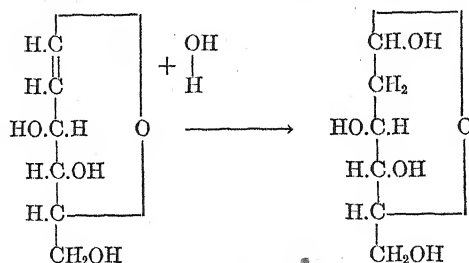
Glucal is transformed to *d*-arabinose on treatment with ozone, incidentally proving that the double bond connects atoms 1 and 2.

It therefore follows that epimeric pairs of sugars will give rise to the *same* glycal, just as they yield the same osazone. Consequently, by the action of perbenzoic acid the epimeric pair of parent sugars is regenerated from any one glycal, the sugar having *cis* hydroxyls at positions 2 and 3 apparently predominating.



Desoxysugars.—The desoxysugars constitute a class of sugar derivatives occurring in nature in biologically important substances.

On treatment of glucal with cold dilute sulfuric acid the elements of water are added at the double bond, a 2-desoxyglucose being formed:



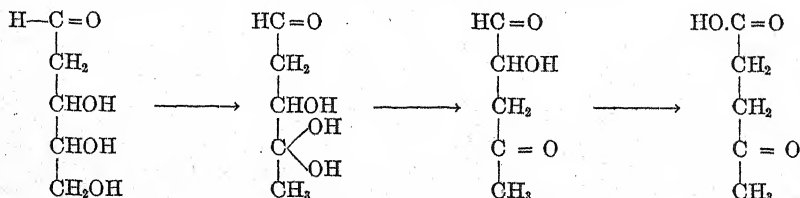
This reaction is the general method for the preparation of *2-desoxy-sugars*.

The 2-desoxyribose and 2-desoxyxylose prepared in this way are of special significance inasmuch as by their aid it was possible to identify the sugar occurring in animal nucleic acid as 2-desoxy-*d*-ribose.²¹

A sugar of this type is also found in digitalis glycosides, namely, a 2,6-didesoxyhexose (or "desoxymethylpentose"):



The general properties of the 2-desoxysugars resemble those of the simple sugars, differing, however, in their inability to form osazones owing to the absence of a hydroxyl group on the second carbon atom. They are very unstable and readily resinify to a green tar. Like ordinary aldehydes, they restore the color to fuchsin-sulfurous acid and in addition they impart a green color to a pine shaving impregnated with a solution (of a desoxy sugar) when it is exposed to water vapor saturated with hydrogen chloride. Levene and Mori found that they are readily converted to levulinic acid by the action of mineral acid and suggest the following mechanism for the reaction:



2-Desoxyglucose reduces Fehling's solution and forms hydrazones but is especially characterized by the extreme ease with which it combines with alcohols to give *glucodesosides*. Thus, it condenses completely with methyl alcohol in the presence of less than 1 per cent of hydrogen

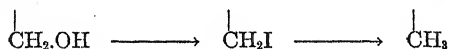
chloride in less than fifteen minutes. The glucodesosides are very easily hydrolyzed to the desoxy sugar. Yet despite these properties, which might be supposed to indicate a furanose structure, Bergmann and Breuers as well as Levene and Mikeska have shown that they are pyranose in structure.

Members of another group of desoxysugars—the ω -desoxysugars—are also of great importance, being widely distributed in nature. Some of the sources from which they have been isolated are given in the table below. They have been erroneously termed methylsugars since the terminal (ω) primary hydroxyl group is replaced by a hydrogen atom thus:



The old terminology is especially unfortunate since it fails to reveal the relationship of the ω -desoxysugar to its parent sugar. Thus, rhamnose should be regarded as 6-desoxymannose, so that it is an ω -desoxyhexose rather than a "methylpentose."

Representatives of ω -desoxy-trioses, -tetroses, -pentoses, -hexoses and -heptoses have all been prepared synthetically either by ascent or descent from the naturally occurring members or by reduction of the ω -iodo-derivatives.



They behave like the monosaccharides, having none of the instability characteristic of the 2-desoxysugars. On heating with hydrochloric acid, they are converted into methyl furfural.

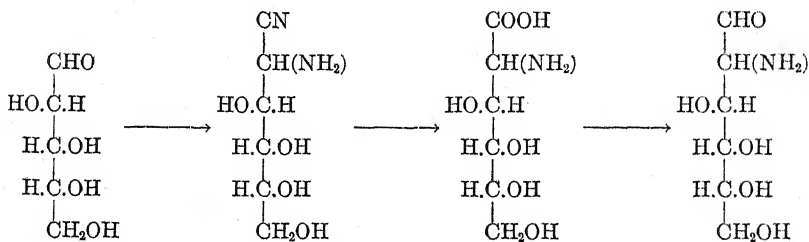
OCCURRENCE OF THE ω -DESOXYSUGARS

| Sugar. | Common name. | Source. |
|---|------------------------|--|
| 3-Desoxyglyceric aldehyde..... | Lactic aldehyde. | The leaves of the poplar tree. |
| 6-Desoxyhexoses, $\text{C}_6\text{H}_{12}\text{O}_5$ 6-Desoxy- <i>d</i> -glucose | <i>d</i> -Isorhamnose. | From purgic acid and the glycosides, convolvulin and chinovin. |
| 6-Desoxy- <i>d</i> -galactose..... | <i>d</i> -Fucose. | In the glycosides, convolvulin and jalopin. |
| 6-Desoxy- <i>l</i> -galactose..... | <i>l</i> -Fucose. | As polymerized anhydride (fucosan) in seaweed (Japanese Nori) and gum tragacanth. |
| 6-Desoxy- <i>d</i> -allose..... | Digitalose. | From digitalin, a digitalis glycoside. |
| 6-Desoxy- <i>l</i> -mannose..... | <i>l</i> -Rhamnose. | As glycosides: Quercetrin, frangulin, hesperidin, rutin, xanthorhamnin, solanin, strophanthin, etc.: as a component of di- and trisaccharides. |

The Aminosugars.—The aminosugars are widely distributed in nature. *d*-Glucosamine¹⁹ is a component of mucins, mucoids, and glucoproteins. It is also the principal organic cell-wall constituent of the fungi and of the hard shell of crustaceae. In the latter it occurs as *chitin*, the polyacetate of a polysaccharide-like substance formed by the union of many glucosamin residues. An analogous substance, a derivative of galactose, is called *chondrosamine*, and is a constituent of gristle.

Glucosamine is most conveniently prepared by hydrolyzing chitin with concentrated hydrochloric acid, the substance being isolated as its hydrochloride.

Both the α - and β -forms of glucosamine hydrochloride, and glucosamine pentacetate are known. Like the glucose derivatives, these forms exhibit mutarotation. Glucosamine was successfully synthesized from *d*-arabinose by Fischer and Leuchs by successive conversion into 2-aminoglucononitrile, 2-aminogluconic acid and reduction of the lactone to *d*-glucosamine:



On treating glucosamine with nitrous acid, both the amino groups and the elements of water are eliminated, giving a sugar named *chitose*, $\text{C}_6\text{H}_{10}\text{O}_5$, which is a 2:5 anhydrohexose.

Glucosamine (chitosamine) may be converted either into glucose or mannose (according to the conditions) by a long series of operations in *one* of which a Walden inversion occurs, so that there has been much dispute as to whether it is the glucose or the mannose derivative. The structure of 2-aminoglucose has been assigned to chitosamine by both Irvine and Levene, and this conclusion has recently been substantiated.

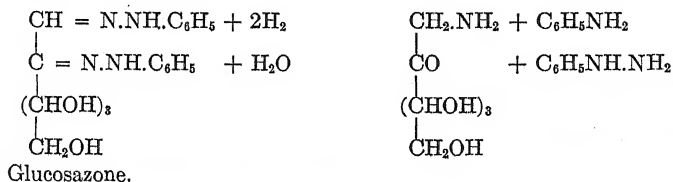
The epimeric form of the sugar was prepared by Levene and provisionally named by him *epichitosamine*. Chitosamine hydrochloride was oxidized to chitosaminic acid, which was converted into epichitosaminic acid by heating with pyridine in an autoclave at 100° C. This substance was lactonized and on reduction gave epichitosamine, isolated as the crystalline hydrochloride.

Under the same name (epiglucosamine) Fischer described a substance obtained by the action of ammonia on 2-chloromethylglucoside. However, Levene has shown this to be a misnomer. Fischer's aminosugar is not the epimer of 2-aminoglucose but has the structure of a 3-aminohexose of unknown configuration.

Another glucosamine (6-aminoglucose) has been prepared by the action of liquid ammonia on triacetyl methylglucoside 6-bromhydrin.

A new method for the synthesis of aminosugars with the amino group in a definite position has been introduced by Freudenberg and his coworkers. This consists in treating the mono-*p*-toluenesulfonyl ester (from the acetone derivative) with alcoholic ammonia. Thus, 3-*p*-toluenesulfonyl diacetone glucose yields 3-aminoglucose, whereas 6-*p*-toluenesulfonyl monoacetone glucose gives 6-aminoglucose.

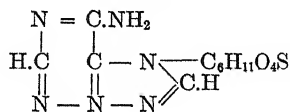
Fructosamine is formed by reduction of its osazone with zinc dust and acetic acid:



This may be reconverted to fructose by treatment with nitrous acid.

Thiosugars.—Two types of thiosugars are known. Those of the first class have the sulfur atom substituted in the hydroxyl group of carbon atom 1. Since they are only known as synthetic products, not having been discovered in nature as yet, they are of purely academic interest. It is claimed that 1-thioglucose occurs naturally in the glycoside sinigrin.

In the other group of thiosugars, the sulfur atom is attached to a carbon atom other than that at position 1 of the chain. A representative of this class, a thiomethylketopentose, occurs in nature as the adenine nucleoside:

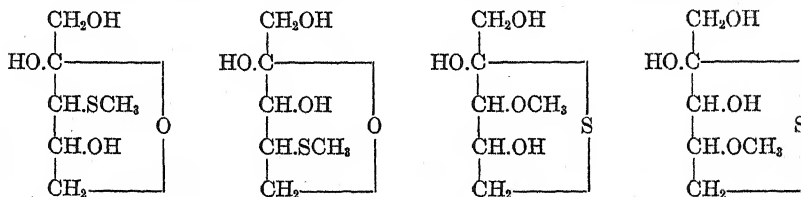


This compound was isolated by Mandel and Dunham from the commercial yeast product *zymin*. They erroneously reported it to contain no sulfur and consequently decided that it was an adenine hexoside, which naturally has the same percentage of carbon and hydrogen as the nucleoside given above. They identified the base as its characteristic picrate and prepared the osazone of the sugar component. On the basis of the melting point of this osazone, they suggested that the sugar was gulose.

Levene recognized the sugar to be a ketose, since it is not oxidized by bromine. He also demonstrated definitely the nonidentity of its osazone with that of gulose.

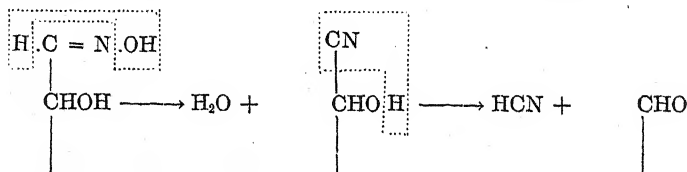
At about the same period it was found by the Japanese workers, Suzuki, Odake and Mori, that the substance contained sulfur. It was therefore reinvestigated by Levene and Sobotka who showed

that the osazone contains sulfur. It follows that the sulfur atom is not situated on the first or second carbon atoms. They also found that the sugar forms a triacetate and has therefore but three free hydroxyl groups. In addition, since distillation with hydriodic acid showed the presence of one OCH_3 or SCH_3 group, the possibility of a 6-carbon chain structure for the sugar was excluded. There thus remain four possibilities for the structure of this thiomethylketopentose:



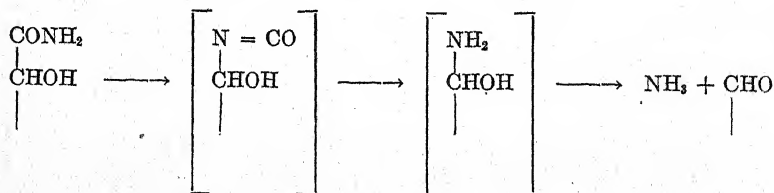
The Degradation of Sugars.—There are three main methods for “descent” of the sugars:

1. *Wohl's Method.*—(a) By treating the oxime of the sugar with alkali, the nitrile is formed and, on heating, loss of hydrogen cyanide occurs to give a sugar containing one carbon atom less:



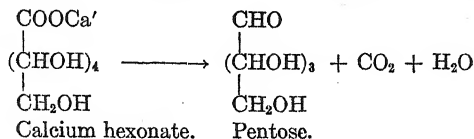
(b) A better method is to heat the oxime with acetic anhydride and zinc chloride (or fused sodium acetate). This removes one molecule of water and at the same time acetylates the hydroxyl groups. The product is then treated with silver oxide to remove the elements of hydrocyanic acid.

2. *Weerman's Method.*—This resembles the Hofmann reaction. The sugar to be degraded is oxidized to the monobasic acid, the lactone of which is treated with dry ammonia to give the amide. On treatment with alkaline sodium hypochlorite, ammonia is liberated and the desired sugar is formed:

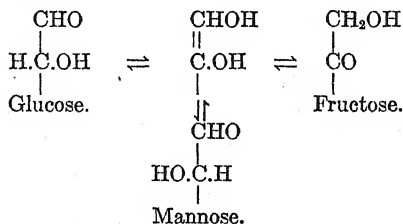


Thus glucose gives arabinose, and galactose yields lyxose. The intermediate forms (indicated in parentheses) are not isolable.

3. *Ruff and Ollendorf's Method.*—The sugar is oxidized to the monobasic sugar acid, the calcium salt of which is treated with Fenton's reagent (hydrogen peroxide in the presence of a ferrous salt).

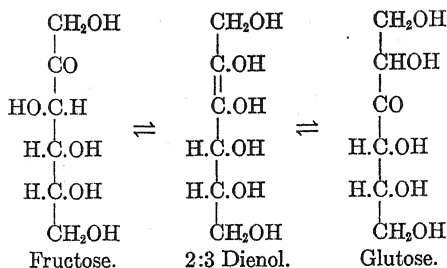


Dissociation or Disruption.—*The Action of Alkalis on Monoses.*—On dissolving the α - or β -form of a sugar in water and adding a trace of alkali the first effect is the production of an equilibrated mixture of the α - and β -forms. On standing in dilute alkali, more extensive changes set in, the first being the "Lobry de Bruyn transformation." As we have previously seen, there is a close interrelationship between glucose, mannose and fructose. Starting with an aqueous solution of any one of those three sugars, the rotation falls to about 0° when alkali is added, the *same* equilibrium mixture of the three sugars being formed in each case. The change takes place slowly at ordinary temperatures, quickly and with considerable decomposition at higher temperatures. Wohl explained this on the assumption of the wandering of a hydrogen atom to give the same intermediate enol body from each sugar. When this suffers the reconversion the hydrogen may assume any one of three positions:

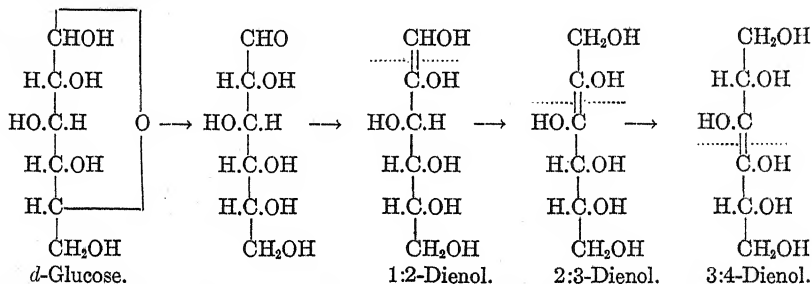


Similarly, d -galactose $\rightarrow d$ -talose + d -tagatose; d -gulose $\rightarrow d$ -idose + d -sorbose. In 1928 Wolfrom and Lewis extended these observations to the methylated sugars. They transformed tetramethyl mannose to tetramethyl glucose, incidentally confirming Haworth's conclusion that the two substances have the same ring structure. Since the hydroxyl group attached to the second carbon atom was methylated, tetramethyl fructose was not formed.

When the sugar solutions are kept with alkali at 37°C. , the optical rotation of the solution decreases and the acidity increases. Besides the 1:2-enol already discussed, a 2:3-enol can obviously form, so that fructose (for example) can be transformed to glucose which Lobry de Bruyn has isolated as a regular product of the transformation of glucose. It occurs to the extent of about 5 per cent in Louisiana molasses.

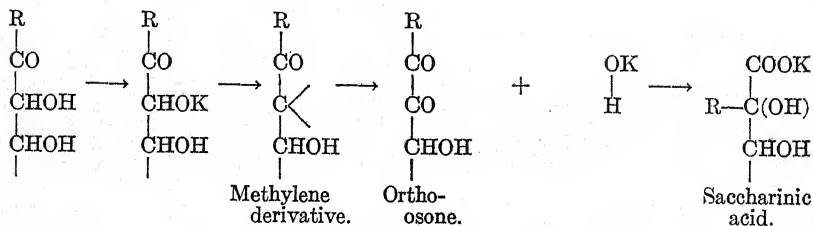


In the presence of air or other oxidant, the tendency is for the unsaturated enols first formed to undergo scission at the double bond. Thus, a hexose will give the 1:2-, 2:3- and 3:4-dienols, which then decompose spontaneously with the formation, respectively, of formaldehyde and an aldopentose; diose and an aldotetrose; and *d*-, *l*-glycerose. The configuration of the resulting pentose and tetrose will naturally depend on that of the parent hexose.

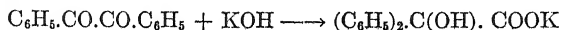


These products then undergo further oxidation to give a mixture of carbon dioxide, formic, glycollic, oxalic and *d*-*l*-glyceric acids, four trihydroxybutyric acids, eight tetrahydroxyvaleric acids, and eight tetrahydroxyhexoic acids, all of which have been isolated and identified.²⁰

In concentrated alkali, in the absence of an oxidant, the sugars give rise to saccharinic acids. Stearn has studied, by the conductivity method, the capacity of the common sugars to combine with alkali and finds that in 2 *N*-alkali the sugars behave as polybasic acids. Salt formation with the alkali hydroxide takes place at the carbon atom next to the carbonyl group, thus:

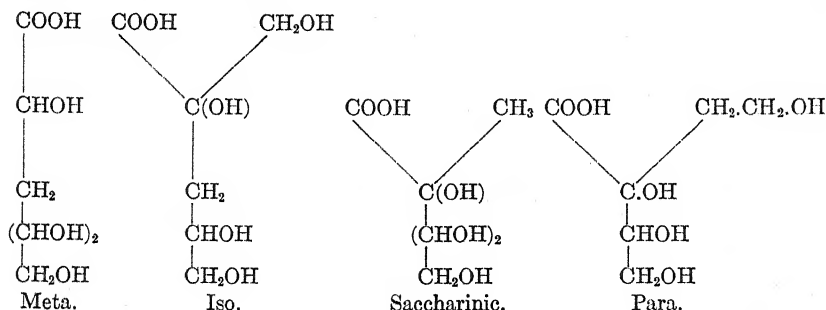


The methylene derivative first forms glycide, then ortho-osone, from which, by the benzilic acid transformation, saccharinic acids are formed.



Nicolet has recently proposed an alternative mechanism for the reaction.

Thus, the sixteen aldohexoses can theoretically give eight stereoisomeric metasaccharinic acids, and the eight ketohexoses can give four isosaccharinic acids. Eight saccharinic acids and four parasaccharinic acids are also possible. Saccharinic acid is formed from glucose and fructose; isosaccharinic acid from the glucose part of maltose, lactose and cellobiose; meta- and parasaccharinic acids from galactose and the galactose part of lactose.



The lactones of these acids are known as *saccharins*.

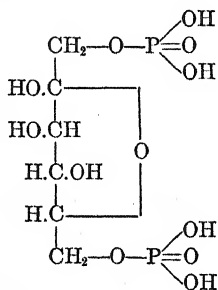
FERMENTATION

A difference in the configuration of one or more carbon atoms in sugars profoundly affects their biochemical behavior. Thus, yeasts only attack the *d*-form of glucose, fermenting it with the formation of carbon dioxide and alcohol. Consequently, when *d*-*l*-glucose is submitted to the action of yeasts the *l*-glucose is recovered unchanged. A comprehensive investigation of all the known hexoses, whether natural or synthetic, reveals that only four are fermented by yeasts. These are the *d*-forms of glucose, mannose, fructose and galactose, all of which are found in nature. In addition, it has been found that some yeasts capable of fermenting any one of the first three sugars will ferment the other two of the three with approximately the same readiness.

Just as, under the influence of alkali, it is presumed that these sugars are mutually interconvertible through the intermediate formation of a common enolic substance, so it has been suggested that the first stage in fermentation is the formation of the enol by means of an enzyme in the yeast. Fermentation then proceeds by some series of degradations ending in the production of carbon dioxide and ethyl

alcohol. The rate of fermentation, which is considerably influenced by the temperature, is conveniently measured by determining the rate of formation of carbon dioxide.

In 1905 it was discovered⁵⁵ that in the fermentation of glucose by zymase a hexose diphosphate, $C_6H_{10}O_4(H_2PO_4)_2$ is first formed. Glucose, mannose and fructose yield the same hexose diphosphate which, on hydrolysis by boiling water, gives fructose. The diphosphate may therefore possibly be formed from the enol. The hexose diphosphate is strongly reducing to boiling Fehling's solution. It reacts with phenylhydrazine in the warm to give glucosazone monophosphate, $(C_6H_5.NH.N)_2.C_6H_6(OH)_3.H_2PO_4$ (isolated as its phenylhydrazine salt); whereas in the cold the product is the phenylhydrazone of the hexose diphosphate, $(C_6H_5.NH.N).C_6H_7(OH)_3.(H_2PO_4)_2$ (isolated as its diphenylhydrazine salt). Hence one of the phosphoryl radicles is presumably attached to the carbon atom adjacent to the "reducing" group in the parent substance, which is probably⁵⁶ fructofuranose-1: 6-diphosphoric acid:

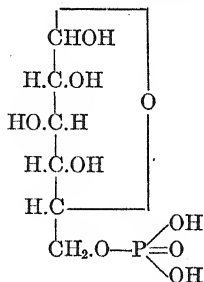


Fructose monophosphoric acid was obtained by Neuberg⁵⁷ by careful hydrolysis of the above diphosphoric acid. Unlike the acid from which it is derived, it is fermented by living yeast. It is also fermented by such dried yeast preparations as zymine. Robison⁵⁸ has described a crude hexose monophosphate, produced by the action of yeast juice on glucose and fructose, which is a mixture of the above fructose monophosphate with an aldohexose monophosphate. The two esters have been separated by fractional crystallization of their brucine salts.⁵⁹ The aldohexose monophosphate was shown to be glucopyranose-6-phosphoric acid.

Levene and Raymond have synthesized glucose-6-phosphate by the phosphorylation of monoacetone glucose and of 1,2,3,4-tetracetyl glucose. The product gives an osazone identical with that obtained from Harden and Young's fructose diphosphate, from Neuberg's fructose monophosphate and from a pure sample of Robison's aldohexose monophosphate. The fermentation rate of this synthetic ester was practically identical with that of Robison's ester.

The fact that Robison's ester, like synthetic glucose-6-phosphate, gives rise to both the furanoside and pyranoside forms of glycoside

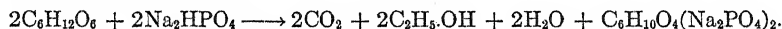
when treated with methyl alcohol at 18° and 60° C. and that the rate of lactone formation of the phosphoaldonic acid obtained by oxidation of the synthetic and natural glucosephosphates is practically identical, both confirm the allocation of the phosphoryl radicle to position 6:



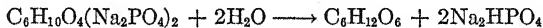
Glucopyranose 6-phosphoric acid.

When glucose, or fructose, is fermented by dried yeast or zymine some 10 to 20 per cent of the total phosphoric acid esterified consists of the monophosphate of trehalose, a nonreducing disaccharide which is also found in yeast, seaweeds, certain fungi and in the "manna" excreted by the insect *Larinus maculatus*.

In 1901 it was first observed⁶⁰ that addition of sodium phosphate increases the total fermentation by yeast juice but it remained for Harden and his coworkers to show the fundamental part played by phosphates in yeast fermentation. It is found that the increase in carbon dioxide is proportional to the amount of phosphate added and that for each mole of carbon dioxide formed one mole of phosphoric acid is esterified. Two molecules of hexose are involved, one being transformed to the hexose diphosphate, the other being degraded to carbon dioxide and alcohol. Thus, ignoring the formation of trehalose and other by-products, the reaction may be regarded as taking place in the following way:



The decomposition of this hexose diphosphate then occurs according to the equation:



and the reactions are repeated, so that no accumulation of inorganic phosphate forms. As cessation of the fermentation sets in, however, the inorganic phosphate does accumulate.

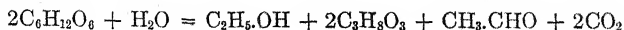
Harden has also shown that the addition of arsenate produces a rapid fermentation which is comparable with that produced by adding phosphate, but it differs from the latter since the rate is permanently raised and no accumulation of sugar phosphate occurs. This is because the phosphatase is made more effective, so that sugar phosphate is hydrolyzed so rapidly that formation of carbon dioxide and alcohol proceed at maximum velocity.

The identification of the intermediate products in the fermentation of glucose has long been a matter of controversy. Pasteur⁶¹ first observed the normal occurrence of glycerol in fermented sugar solutions, the alcohol and carbon dioxide formed amounting to only about 95 per cent of the sugar fermented. It has been shown⁶² that when sodium sulfite is added to a sugar solution in which yeast is growing, acetaldehyde-sodium bisulfite, $C_2H_4O \cdot NaHSO_3$, is readily isolated. It is presumed that acetaldehyde is an intermediate product which is "fixed" and removed from the scene of reaction by the bisulfite. At the same time, an equimolecular proportion of glycerol is formed. This evidence would indicate that a triose derivative, methyl glyoxal (pyruvic aldehyde), or one of its possible modifications, which is capable of oxidation to pyruvic acid on the one hand or of reduction to glycerol on the other, is preformed. The sugar molecule is presumed to break down into two molecules of pyruvic acid, $CH_3 \cdot CO \cdot COOH$, which are readily converted into acetaldehyde by the yeast carboxylase.

In the presence of alkalis,⁶³ the aldehyde formed in the fermentation undergoes dismutation with the formation of equal molecular proportions of acetic acid and alcohol by a Cannizzaro reaction:



Just as in the fermentation in presence of sulfite, the withdrawal of the aldehyde from the system involves the production of a molecular equivalent of glycerol so that the equation for this type of fermentation is:

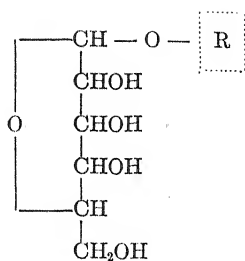


THE DISACCHARIDES

The second group of the carbohydrates, the disaccharides, contains compounds which may be regarded as the products of the condensation of two monosaccharides with the elimination of one molecule of water and which therefore have the general formula $C_n(H_2O)_{n-1}$.

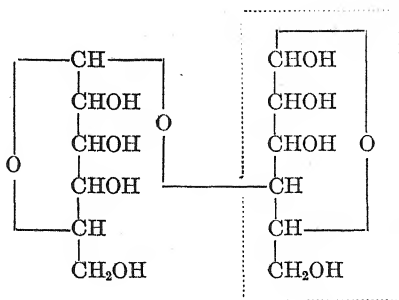
Two classes of disaccharides are recognized, the reducing and the nonreducing; and these may obviously be distinguished by their behavior toward Fehling's solution.

Reducing Disaccharides.—These disaccharides may be regarded as glycosides of the part (A), the alkyl residue (R) in (I) being replaced by a monosaccharide (B), which, in its turn, has the aldehydic group free. Thus the portion (B) possesses all the properties of a monosubstituted monose and (A) all the properties of an ordinary glycoside. Group (B) possesses reducing power, is capable of existence in both α - and β -forms and is able to form glycosides with other alcoholic groups. These glycosides may possess different ring structures, the character of their ring structure being determined by the position of the substituting monose inasmuch as the substituted hydroxyl group is no longer capable of taking part in a semiacetal linking with the aldehydic group.



A.
Alkyl glucoside.

I.



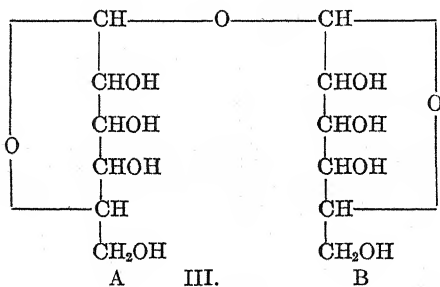
A. B.
Glucose. Glucose.
Cellobiose.

II.

The residue (A) has lost its reducing power and can exist, in different disaccharides, either in α - or β -form; but for each disaccharide the form is fixed. Also the residue (A) may exist in different disaccharides in different cyclic forms but again, for a given disaccharide, the ring form is fixed.

Hence, for each disaccharide, the fixed characteristics are: First, the position of the substituting monose attached to residue (B); second, the α - or β -stereochemical form of the (A) component; and third, the ring structure of the (A) component. Individual disaccharides composed of identical monosaccharides may differ in one or all of these respects and in order to establish the structure of a reducing disaccharide, it is necessary to secure information on these three points.

Nonreducing Disaccharides.—These disaccharides may be regarded as glycosides in which the alkyl residue (R) attached to residue (A) [I] is replaced by a monose (B) [III] by attachment through its reducing group. Thus *both* components should possess all the properties of an ordinary glycoside. Both residues have lost their reducing power and each can exist, in different disaccharides, in α - or β -form. But in each disaccharide the form of both component monoses is fixed. Thus, there are three possible nonreducing diglucoses: α -Glucose + α -glucose; α -glucose + β -glucose; and β -glucose + β -glucose.



A B
III.

Where the two monoses are not identical, four stereochemically different disaccharides are theoretically capable of existence; for example, α -glucose + α -fructose; β -glucose + α -fructose; α -glucose + β -fructose; and β -glucose + β -fructose.

Both residues may exist in different ring forms, but again for any given disaccharide, the ring form is fixed. Hence, for each nonreducing disaccharide the fixed characteristics are first, the α - and β -stereochemical configurations of both components, and secondly, the cyclic form of both residues. In order to determine the structure of a non-reducing disaccharide, it is necessary to obtain information on these two points.

Four principal methods have been used to arrive at the **constitution of the disaccharides**:

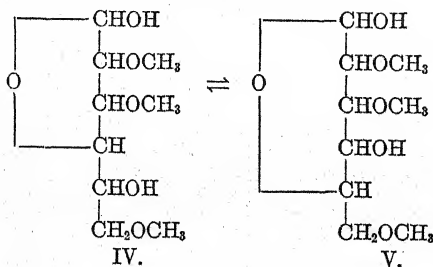
1. *The Methylation Method*.—Methylation of the nonreducing disaccharides may be carried out by the agency of silver oxide and methyl iodide, or by digestion in aqueous solution with methyl sulfate and alkali. In this way the octamethyl disaccharide is obtained. By the hydrolytic cleavage of these fully methylated products, two methylated sugars are obtained. Thus,

Nonreducing disaccharides \rightarrow 2 tetramethyl hexoses.

Reducing disaccharides \rightarrow (A) tetramethyl hexose + (B) trimethyl hexose.

In all the reducing disaccharides so far examined, the hydrolytic component (A) has always been found to be either 2:3:4:6-tetramethyl gluco- or 2:3:4:6-tetramethyl galactopyranose. These two sugars are crystalline, and are readily characterized by means of their anilides, hydrazones, etc.

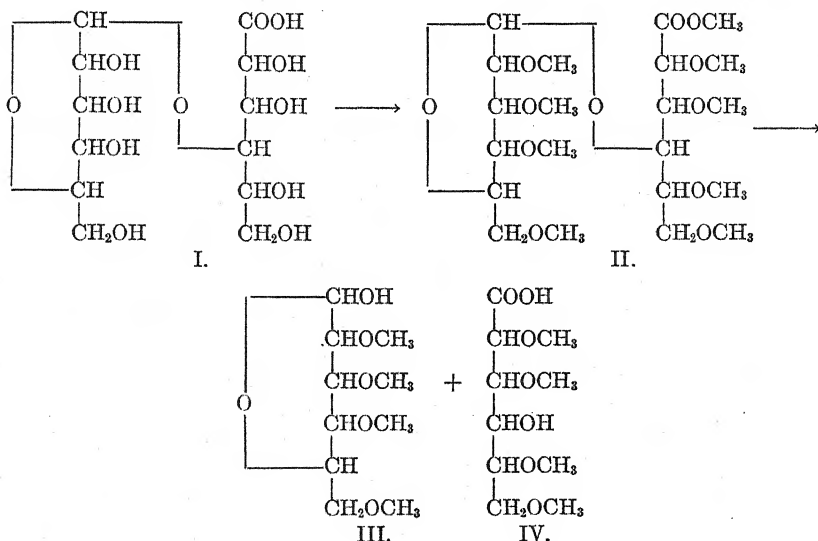
The trimethyl sugar (B) is not so readily identified and, in addition, the position of the linkage in the biose is left in doubt, since there are two unsubstituted hydroxyl groups in this sugar molecule. For this reason the application of this method to the reducing disaccharides does not yield conclusive evidence of structure. For example, if the original disaccharide were a 5-substituted glucofuranose or a 4-substituted glucopyranose, the *same* 2:3:6-trimethyl glucose would be obtained on methylation and hydrolysis, since the 2:3:6-trimethyl glucofuranose (IV) primarily existing in the former case can readily change into 2:3:6-trimethyl glucopyranose (V).



2: 3: 6-Trimethyl glucose is easily identified in the pure state when it is crystalline, but Irvine has shown that impurities interfere with its crystallization so that it may be confused with the uncrystallizable 2: 3: 4-trimethyl glucose. Further, traces of impurities greatly change the melting point and specific rotations of methylated sugars.

2. *Methylation of the Bionic Acids*.—In order to overcome the difficulties offered by the isolation and identification of a trimethylhexose from the hydrolytic products of the methylated reducing disaccharides, Haworth²¹ has resorted to the methylation of the bionic acids.

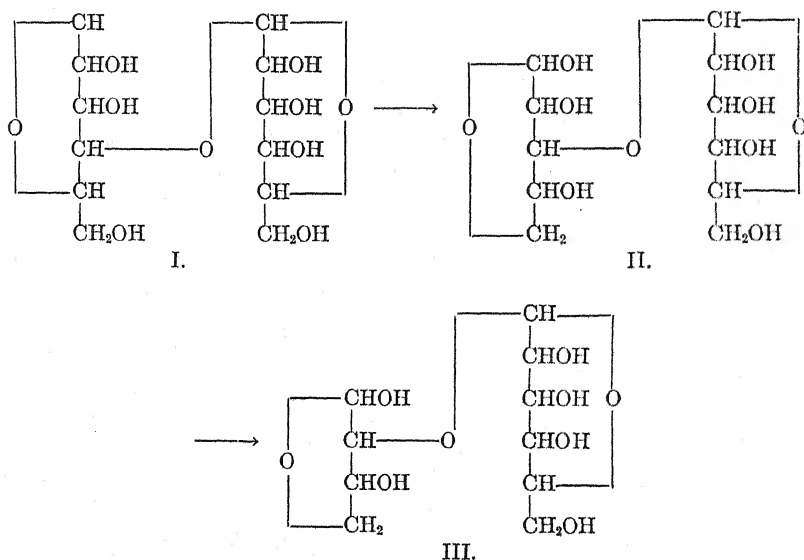
All the reducing disaccharides may be oxidized to the corresponding monobasic acids, with the rupture of one oxygen ring. Methylation of the bionic acid (I) so produced gives the octamethyl methylbionate (II), which yields on hydrolysis a tetramethyl hexose (III) and a tetramethyl hexonic acid (IV):



This tetramethyl hexonic acid has only one unsubstituted hydroxyl group, the position of which corresponds to the linkage position in the original acid. The identity of this position can be ascertained by observing the rate of lactonization. A δ -lactone will be formed when the hydroxyl group attached to carbon atom 5 is free, whereas a γ -lactone will be formed if the hydroxyl group attached to carbon atom 4 is free. (See method (4) below.)

3. *Degradation Method*.—Zemplén²² has used a fundamentally different method to decide the point of union of the two hexose units in a reducing disaccharide. By degrading the reducing component till it no longer forms an osazone, he has determined the constitution of lactose, maltose, cellobiose, and other sugars.

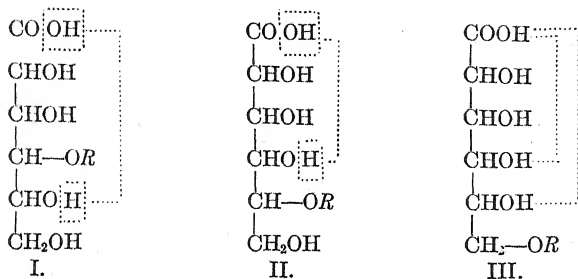
In the case of lactose (I) the oxime is prepared by treatment of the sugar with hydroxylamine and from this the nitrile is obtained. Degradation of the nitrile with sodium methylate yields *d*-galacto-*d*-arabinose (II). By repetition of this process the latter disaccharide gives *d*-galacto-*d*-erythrose (III), a sugar which does not form an osazone. The oxygen bridge between the galactose residue and erythrose component must therefore be at carbon atom 2 in the erythrose chain and hence at carbon atom 4 in the original glucose chain.



4. *The Lactone Formation Method.*—Levene and Simms²³ showed that during lactonization of those monobasic sugar acids which have free hydroxyl groups in positions 4 and 5, two lactones are simultaneously formed in aqueous solution. These differ in their rates of formation and in the percentage of each lactone present when equilibrium with the free acid has been attained in aqueous solution. The δ -(1:5) lactone forms rapidly and at the point of equilibrium the lactone has a concentration of 20–30 per cent of the original acid. The γ -(1:4) lactone forms slowly and at the point of equilibrium the lactone has a concentration of 75–80 per cent of the original acid.

It has been shown that the reverse process holds, *i. e.*, in lactone hydration the δ -lactones undergo a rapid and extensive transformation whereas with γ -lactones the formation of free acid is slow and incomplete.

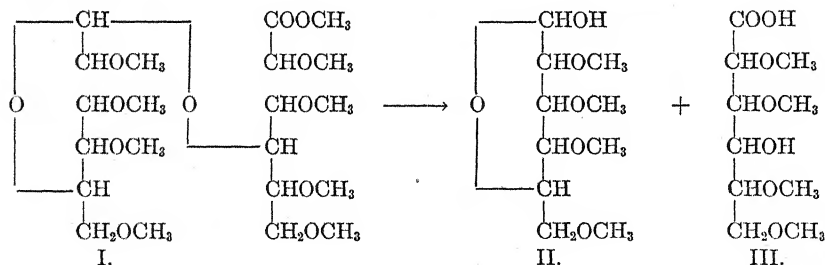
In substituted sugar acids (*e. g.*, the bionic acids), it is therefore possible to determine whether the substituent hexose residue *R*, is in the 4 or 5 position. (See I, II and III below.) The method has been applied to lactose, maltose, cellobiose, and melibiose by Levene.



Cellobiose.—The structure of cellobiose is of importance since it is derived from cellulose by acetolysis, *i. e.*, simultaneous acetylation and hydrolysis. It has not been found to occur in nature in the free state. It reduces Fehling's solution and gives methylcellobioside with methyl alcohol containing hydrogen chloride.

Hydrolysis of heptamethyl methylcellobioside yields a mixture of 2:3:4:6-tetramethyl glucopyranose and 2:3:6-trimethyl glucopyranose. Since 2:3:6-trimethyl glucopyranose may have preexisted in the disaccharide as 2:3:6-trimethyl glucofuranose, two structural formulae are possible for cellobiose on the basis of this evidence—the union of the two hexose components may have been at carbon atoms 4 or 5.

However, hydrolysis of octamethyl methylcellobionate (I) yields a mixture of 2:3:4:6-tetramethyl glucopyranose (II) and 2:3:5:6-tetramethyl gluconic acid (III). As will be seen from the formulae below, it follows that the hexose residues must be united through carbon atom 4.



Maltose.—Hydrolysis of heptamethyl methylmaltoside leads to a mixture of 2:3:4:6-tetramethyl glucopyranose and 2:3:6-trimethyl glucose. The results obtained on methylation and lactonization of maltobionic acid and by the Zemplén degradation of maltose show that maltose and cellobiose are stereo-isomers. Hence, maltose is 4- α -glucosidoglucopyranose and cellobiose is 4- β -glucosidoglucopyranose.

Gentiobiose.—Hydrolysis of heptamethyl methylgentiobioside yields 2:3:4:6-tetramethyl glucopyranose and 2:3:4-trimethyl glucopyranose. Thus, the gentiobiose must be 6- β -glucosidoglucose and this structure has been confirmed by synthesis.

Lactose.—Methylation of lactose followed by hydrolysis yields tetramethyl galactopyranose and 2:3:6-trimethyl glucose. That it has the structure of 4- β -galactosidoglucose has been confirmed by degradation to a galacto-erythrose which forms no osazone; further, by the formation of a δ -lactone from lactobionic acid, and the production of tetramethyl- δ -gluconolactone. Furthermore, the galacto-arabonic acid derived from lactose (on degradation) forms both a γ - and a δ -lactone.

Melibiose.—In 1919 Haworth and Leitch suggested that melibiose was 6- β -galactosido-glucose, by analogy with the structure they had at that time incorrectly assigned to maltose (6- α -glucosidoglucose). Besides the normal tetramethyl galactopyranose, 2:3:4-trimethyl glucopyranose was isolated from the hydrolytic products of methylated melibiose. Oxidation of the tetramethyl gluconic acid obtained from melibionie acid gives a tetramethyl saccharic acid. Helferich synthesized 6- β -galactosidoglucose and the synthetic product proved to be entirely different from melibiose. By comparing the specific rotations of melibiose and its derivatives with the specific rotations of α - and β -disaccharides and their derivatives, evidence was obtained that melibiose is in reality an α -galactoside.

Evidence against the 6-position of union has been advanced by Zemplén, who degraded melibiose and obtained a galactosidopentose, which did not form an osazone. More recently, however, Levene and Jorpes have confirmed the 6- α -galactosidoglucose formula for melibiose by obtaining both the required γ - and δ -lactones from meliobionic acid. The final confirmation of the structure of melibiose has been afforded by its synthesis by Helferich.

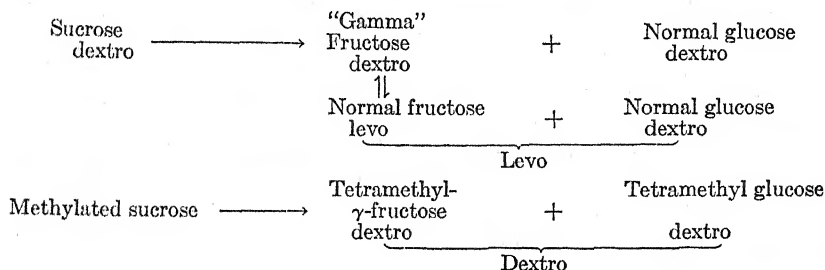
Sucrose.—Since sucrose does not reduce Fehling's solution, the union between the two hexose constituents must be through their reducing groups.

In 1903 Purdie and Irvine prepared methylated sucrose by the aid of silver oxide and methyl iodide. On hydrolysis they obtained crystalline tetramethyl glucopyranose and an uncrystallizable fraction which they took to be the tetramethyl derivative of fructopyranose. They noted further that the uncrystallizable fructose part of the hydrolytic products had a small dextrorotation, whereas the crystalline tetramethyl fructopyranose prepared by Purdie and Paul had a large levorotation.

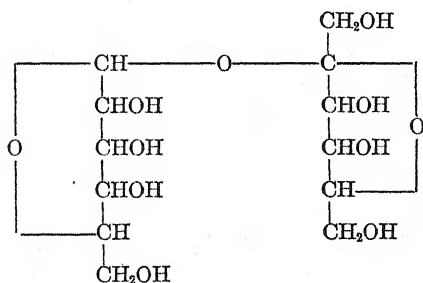
Purdie and Paul²⁴ obtained evidence that in the mixture obtained by preparing methylfructoside by Fischer's method there existed, along with the usual α - and β -modifications, a third form which was less levorotatory and possibly dextrorotatory—a striking prediction. In 1914 Fischer²⁵ isolated a third or "gamma" methylglucoside which was characterized by its extreme ease of hydrolysis and a small levorotation.

To account for the ease of hydrolysis of sucrose, Irvine, Fyfe and Hogg²⁶ postulated that the fructose portion of sucrose had a

similar "gamma" structure. The expected final value for the rotation of the hydrolytic products of methylated sucrose is -18° (calculated from the known values for the methylated hexoses) whereas that actually recorded is $+57^\circ$. This divergence cannot be credited to the glucose component since tetramethyl glucopyranose has been isolated from the cleavage products. Haworth and Law were able to calculate the approximate specific rotation of the liquid tetramethyl fructose and arrived at a value of $+29.3^\circ$. When this value is inserted alongside the equilibrium rotation of tetramethyl glucose ($+83^\circ$) it is seen that the equimolecular mixture of these two methylated hexoses would, in all probability, show a rotation of $+57^\circ$. Haworth²⁷ isolated pure tetramethyl fructofuranose from the scission products of methylated sucrose and found its rotation to be $+31.7^\circ$. Thus the optical inversion of sucrose and the noninversion of octamethyl sucrose can be explained in the following manner:



Much work was performed before the ring structure of the "gamma" fructose constituent of the sucrose molecule could be decided. It was not until 1926 that the problem was finally settled by Haworth and his collaborators who showed definitely by oxidation methods that the "gamma" fructose had the furanose structure. Thus, sucrose may be written as follows:



The stereochemistry of sucrose has not as yet been definitely worked out. The glucose component has been recognized as indubitably having the α -configuration (on the basis of its mutarotation behavior on inversion). The fructofuranose component has an almost instantaneous mutarotation and Haworth and Law have assumed that

it has the α -configuration. Morgan has indicated that the α -form of the methylglycoside of hexosediphosphoric acid—whose carbohydrate portion seems to be constituted similarly to the fructofuranose portion

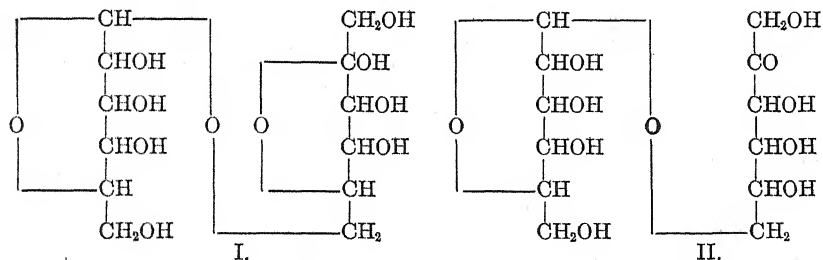
THE NATURALLY-OCCURRING DISACCHARIDES

| Disaccharide. | Source. | M.P. degrees. | Specific rotation (in water) degrees. |
|--|--|---|---|
| 1. Glucoxylose..... | Leaves and branches of <i>Daviesia latifolia</i> . | | —36.5 |
| 2. Arabobiose..... | Arabinic acids. | | +198.8 |
| 3. Strophanthobiose (mannorhamnose). | In the strophanthus glycosides (only known as the methyl ether). | | |
| 4. Rutinose (rham- nosidoglucose).. | In the glycosides, ru- tin and datisein. | | + 3.24→-0.81 |
| 5. Primverose (xylo- sidoglucose).... | In the glycosides, pri- mocerin, gentiarau- lin, rhamnicoside. | 210 | +24.14→-3.3 |
| 6. Vicianose (<i>l</i> -arab- inosidoglucose).. | In the glycosides gein, vicianin, etc. | 210 | +56.5→+39.72 |
| 7. Trehalose (α -glu- cosido - α -glu- cose)..... | In fungi; as the poly- merized anhydride (trehalum) in ergot and molds. | 203 | +197.1 |
| 8. Maltose (4- α -glu- cosidoglucose)... | Degradation of starch. | 160-165 | +118.0→+29.0 |
| 9. Gentiobiose (6- β - glucosido - glu- cose)..... | In the glucoside, am- ygdalin, and the tri- saccharide gentian- ose; gentian roots. | α form: 85.5-86 β form: 190-195 | α : +31.0→+9.6 β : -11.0→+9.6 |
| 10. Dextrinose (iso- maltose)..... | In beer, urine, blood, honey, liver, etc. | | +141.4 |
| 11. Cellobiose (4- β - glucosidoglu- cose)..... | Degradation of cellu- lose. | 225 | +16.0→+35.0 |
| 12. Isocellobiose..... | Degradation of cellu- lose. | 155-165 | +24.6 |
| 13. Sucrose (cane su- gar)..... | In fruits and other parts of plants. | 180-184 | +66.37 |
| 14. Turanose (6- α - glucosidofruc- tose)..... | In the trisaccharide, melezitose. | 60-65 | +43.5→+75.6 |
| 15. Lactose (4- β -gal- actosidoglucose). | In the milk of all mammals. | α form: 201 β form: 252.4 | +80.67→+51.78 |
| 16. Melibiose (6- α -gal- actosidoglucose) | In the trisaccharide, raffinose. | 92-95 | +110.5→+126.5 |

of sucrose—but not the β -glycoside, is partially hydrolyzed by *invertase* which suggests an α -fructosidic constituent in sucrose, since *invertase* also hydrolyzes sucrose,

Turanose.—Turanose is a reducing disaccharide and has been characterized as a glucosido-ketose. Methylation of turanose followed by hydrolysis yields tetramethyl glucopyranose and 1-3-4-trimethyl fructofuranose. Thus turanose is claimed by Leitch to be 6-glucosidofructofuranose (I) though obviously 1:3:4-trimethyl fructose is anomalous, like 2:3:6-trimethyl glucose, and may exist in a pyranose or furanose form, so that the oxygen linkage may be at position 5 or 6. It has been shown that the enzyme which splits turanose is identical with α -glucosidase and therefore turanose is an α -glucosidic disaccharide.

Pacsu has isolated several acetates of turanose and has shown that one of them can be catalytically reduced to a mixture of acetylated alcohols. Had the turanose possessed a ring structure in the fructose component, no reduction could have taken place as the reducing group would have been protected by an acetyl residue. Pacsu therefore suggests that the reducible form is in reality a free keto sugar (II).



DISACCHARIDE SYNTHESIS

Enzymatic Synthesis.—As long ago as 1899 Croft Hill synthesized maltose by the action of yeast on a concentrated glucose solution. This synthesis produced much controversy but it has been finally confirmed. Gentiobiose has been synthesized by means of a yeast containing the enzyme *gentiobiose*. Bourquelot has synthesized gentiobiose, cellobiose, mannobiose and two galactobioses using the enzyme emulsin. Fischer and Armstrong obtained an isolactose by the action of *kefir*-ferments on an equimolecular mixture of glucose and galactose.

Thermal Condensation.—Pictet²⁸ has claimed in recent years to have succeeded in synthesizing a number of disaccharides by the thermal condensation of anhydromonosaccharides. Such syntheses throw no light on the mechanism of formation nor do they permit of any prediction as to the nature of the resulting disaccharide. As a rule the product is not homogeneous.

Chemical Synthesis.—Fischer²⁹ was the first to synthesize a disaccharide by purely chemical means when he obtained isomaltose by the action of hydrochloric acid on glucose in the cold.

When completely substituted monosaccharides (having only the first hydroxyl group free) are condensed, the product must be a non-

reducing disaccharide. But even in this case the product need not be a single substance, as there is the possibility of various stereo-isomeric arrangements. For example, Fischer and Delbrück³⁰ condensed two molecules of acetobromoglucose in the presence of silver carbonate and obtained a mixture of β - β -isotrehalose and α - β -isotrehalose.

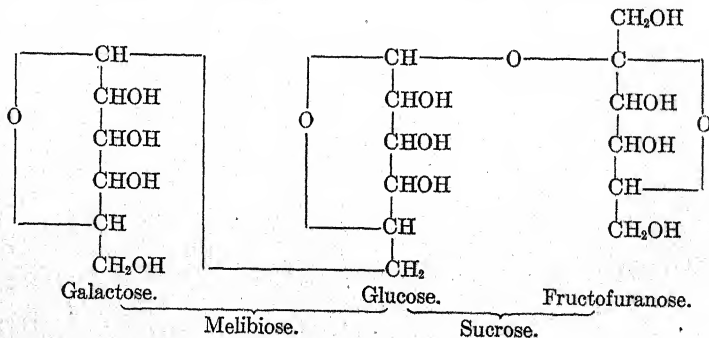
Helferich³¹ has prepared hexose derivatives having the hydroxyl group in position 6 unoccupied, by substituting the remaining hydroxyls in 6-trityl glucose and subsequently removing the trityl residue by acid hydrolysis. By condensing the 1:2:3:4-tetra-acetyl glucose prepared in this way with acetobromoglucose he obtained octa-acetyl gentiobiose. By similar means he was able to synthesize melibiose, 6- β -galactosidoglucose and the naturally occurring glycosides primeverose (6- β -xylosidoglucose) and vicianose (6- β -*l*-arabinosidoglucose).

Freudenberg³² condensed diacetone galactose (which has one free hydroxyl group at position 6) with acetobromogalactose and obtained a 6-galactosidogalactose. An attempt to use diacetone glucose in a similar synthesis failed, presumably owing to steric reasons. Freudenberg has synthesized the following di- and trisaccharides by analogous methods: 6- β -Cellobiosidogalactose- α , 6- β -lactobiosidogalactose, 6- β -galactosido- β -galactose, 6-mannosidogalactose- α , and mannosidomannose.

Synthesis of Sucrose.—Many attempts have been made to synthesize sucrose but failure has so far attended all efforts. In 1928 Pictet and Vogel³³ claimed to have synthesized the octa-acetate of this disaccharide by the condensation of the tetra-acetyl derivatives of glucopyranose and fructofuranose, eliminating water with phosphoric anhydride. Various investigators³⁴ have repeated this experiment and failed to isolate the disaccharide.

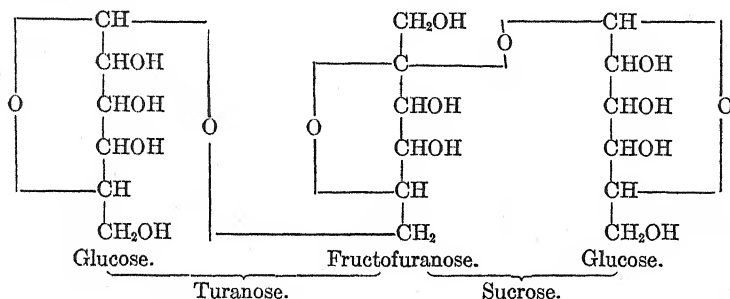
TRISACCHARIDES

Raffinose consists of one residue each of glucose, galactose and fructose and it may be split enzymatically or by chemical means into either fructose and melibiose; or galactose and sucrose. Thus its structure must be α -melibiosidofructofuranoside and α -galactosidosucrose.



Gentianose.—Gentianose is an α -gentiobiosidofructofuranoside or β -glucosidosucrose. Therefore it is represented by the above structural formula providing the galactose residue be replaced by glucose.

Melezitose.—Melezitose is turanosido- α -glucoside or α -glucidosucrose, having the following structural formula:



NATURALLY-OCCURRING TRISACCHARIDES

| Trisaccharide. | Source. | M.P. degrees. | Specific rotation (in water) degrees. |
|--|---|---------------|---------------------------------------|
| 1. Rhamninose (1 mol. galactose + 2 mols. rhamnose)..... | In the glycosides, sophorin and xanthorhamnin. | 135-140 | -41.0 |
| 2. Robinose (1 mol. galactose + 2 mols. rhamnose)..... | In the glycoside, robinin. | | +5.17→1.94 |
| 3. Manninotriose (1 mol. glucose + 2 mols. galactose)..... | From the tetrasaccharide, stachyose (ash-tree manna). | 150 | |
| 4. Levidulinose (2 mols. mannose + 1 mol. glucose)..... | In Konjak, mannan. | | -15.4 |
| 5. Raffinose..... | In sugar beet and cotton seed. | 118-119 | +123 |
| 6. Gentianose..... | In gentian roots. | 209-211 | +33 |
| 7. Melezitose..... | In the manna of Douglas firs, honeydew. | 155 | +88.7 |

THE POLYSACCHARIDES

The group of polysaccharides contains substances having a number of monosaccharide residues condensed together. They are of high molecular weight and form colloids with water, with the exception of cellulose which is completely insoluble. They do not reduce Fehling's solution and are hydrolyzed to monosaccharides by acids. The polysaccharides may be divided into three classes, the pentosans ($C_5H_8O_4$)_x,

the hexosans $(C_6H_{10}O_5)_x$, and mixed polysaccharides. In the latter group, those which contain uronic acid residues in combination with hexose residues occupy a special and important place.

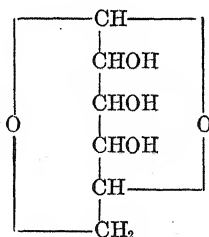
Few derivatives of polysaccharides have been obtained in the pure state. The most productive field for the investigation of their constitution is found in the study of the many scission products to which they give rise under various conditions.

There are two main theories regarding the constitution of the polysaccharides:

1. The first theory is based on the assumption of a polymerizing unit of definite constitution which undergoes molecular association by means of residual valencies to form "high molecular compounds." Such polymerized compounds, under a variety of external conditions, are assumed to undergo molecular dissociation and revert to the original polymerizing unit. Exponents³⁵ of this theory therefore attempt to isolate such molecular units of each polysaccharide. Several sugar derivatives do undergo polymerization with remarkable ease, but the products do not bear any close resemblance to the natural polysaccharides.

By the vacuum distillation of starch, or by heating starch with glycerol to a high temperature,³⁶ a number of anhydrosugars are produced, but their bearing on the structure of the natural polysaccharides has never been demonstrated.

When one such depolymerized unit, β -glucosan, is heated to a high



temperature in the presence of zinc chloride, it undergoes repolymerization to high molecular compounds, none of which has, however, been shown to bear any close relation to starch.

Schardinger and Pringsheim have isolated a series of well-defined amyloses $(C_6H_{10}O_5)_x$, by the action of *B. macerans* on starch, *e. g.*, they have claimed to have isolated di-, tri-, tetra-, hexa- and octa-amyloses, and Karrer found that tetra-amylose is quantitatively converted into acetobromomaltose.

Molecular weight determination on polysaccharides and their derivatives have been carried out by innumerable methods. The results of such determinations have given extremely variable conclusions and no reliance can be placed on such observations. The reason for these variable results is the unreliability of the methods of molecular weight

determination when applied to substances of high molecular weight having colloid properties and is, in some cases, due to the contamination of the substances with impurities. Many speculations regarding the structure of the polysaccharides have been based on evidence derived from such molecular weight determinations but have been shown to be without foundation.

2. The proponents of the second theory consider the polysaccharide molecule to consist of a chain of monosaccharide residues joined by glycosidic linkages. The evidence on which this conception of the polysaccharide molecule is based is as follows: (a) The isolation by Bertrand, Willstätter, and recently by Zechmeister and Toth³⁷ of reducing di-, tri- and tetrasaccharides from the products of partial hydrolysis of polysaccharides by means of concentrated hydrochloric acid; (b) the isolation of methylated di-, tri- and tetrasaccharides from the products of partial degradation of fully methylated polysaccharides by the agency of acetyl bromide by Freudenberg³⁸ and Haworth^{39, 40} and their collaborators; and (c) the isolation of small quantities of a reducing tetramethyl hexose from the products of complete hydrolysis of fully methylated polysaccharides by Haworth and his co-workers.^{40A, 43} On the chain-conception of the polysaccharide molecule such tetramethylated derivatives represent the terminal units of a chain of monoses linked by glycosidic linkages.

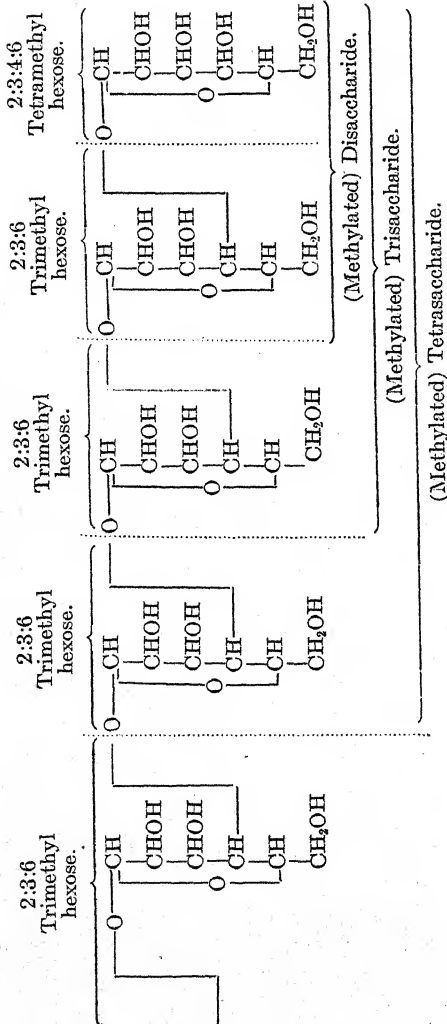
A graphic representation is given on page 96 showing how these various cleavage products could be formed from a polysaccharide having a chain structure.

It must be pointed out that the validity of these results lies in the assumption that no hydrolysis of the polysaccharide molecule takes place prior to the final hydrolysis of the fully methylated polysaccharide. In many cases, methylated polysaccharides have been prepared by methylation of the corresponding triacetyl derivatives. Under certain conditions, the polysaccharides are degraded by the process of acetylation, *e. g.*, by the process of "acetolysis" (simultaneous acetylation and hydrolysis) starch gives maltose octa-acetate and cellulose gives cellobiose octa-acetate. Thus, there is the possibility that the tetramethyl hexose found in the product of the hydrolysis of the fully methylated polysaccharides may be due to partial hydrolysis of the polysaccharide molecule prior to methylation.

Irvine emphasizes the fact that polysaccharides which have been acetylated may have become depolymerized and places no reliance on evidence deduced from polysaccharides which have been pretreated in this way. This view has been supported by Bell who has demonstrated the case of a polysaccharide which had been acetylated and gave tetramethyl glucopyranose on hydrolysis of the methylated product whereas the nonacetylated methylated polysaccharide gave no such hydrolytic product.

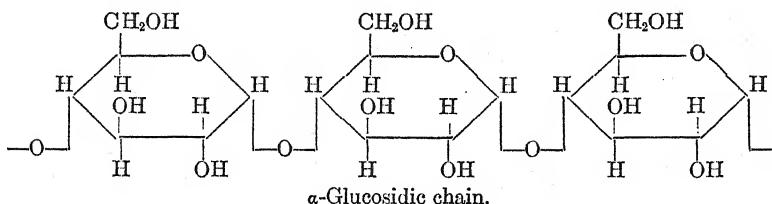
The present position of our knowledge of the structure of the polysaccharides is dependent on and requires a rigid proof of the preexist-

SCHEME FOR THE HYDROLYTIC CLEAVAGE OF POLYSACCHARIDES

(Methylation):
Complete
hydrolysis.(Methylation):
Partial
hydrolysis.

ence in the polysaccharide of a hexose residue which is capable of giving tetramethyl hexose on methylation followed by hydrolysis.

Starch.—Starch consists essentially of two constituents—amylose and amylopectin—which Hirst and his associates⁴¹ consider to have the same chemical structure, differing only in their micellar structure. Both amylose and amylopectin yield glucose exclusively on acid hydrolysis. On acetylation starch yields a triacetate; but by a modification of the procedure simultaneous acetylation and hydrolysis can be induced. By this means 80 per cent of the starch can be obtained as acetylated maltose—4- α -glucosido-glucose. On the basis of this evidence, it would appear that starch consists of glucose residues in α -glucosidic (or maltosidic) linkage as shown below, *i. e.*, α -glucose residues joined between position 4 of one residue and the reducing group of the following unit.

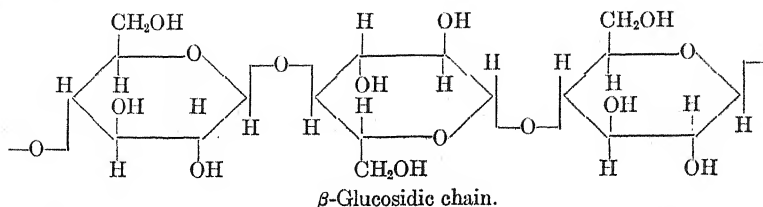


Haworth and Percival³⁹ have degraded methylated starch by means of acetyl bromide and have isolated from the cleavage products di-, tri-, and tetrasaccharides. Complete hydrolysis of fully methylated amylose and amylopectin gives mainly 2:3:6-trimethyl glucose and a small quantity of 2:3:4:6-tetramethyl glucopyranose. On the basis of the final analysis of the amount of 2:3:4:6-tetramethyl glucose in the cleavage products of fully methylated amylose and amylopectin, Hirst and his coworkers⁴¹ have estimated that the number of monosaccharide units in the amylose and amylopectin molecules is about 24, giving both constituents of starch a molecular weight of approximately 5000.

The cyclic form of the glucose residues in amylose and amylopectin has been shown to be pyranoid on the evidence of the formation of maltose octa-acetate as the main product of acetolysis. It is known that maltose is 4- α -glucosidoglucose and consists of two pyranoid glucose residues in a α -glucosidic linkage. It has indeed been claimed that maltose is a "reversion" product from an intermediate hydrolytic product of starch and a furanose structure has been suggested on these grounds, but no chemical evidence exists for the retention of such a structure. Hibbert and Percival, on the other hand, have brought forward convincing evidence in favor of the pyranoid structure by demonstrating the similarity of the rates of hydrolysis of starch and maltose.

Cellulose.—Hydrolytic cleavage of cellulose yields glucose exclusively, and on acetolysis it gives a yield of 50 per cent of cellobiose octa-

acetate (4- β -glucosidoglucose) thus indicating that cellulose is built up of glucose units in cellobiosidic linkage, *i. e.*, β -glucose residues joined between position 4 of one residue and the reducing group of the next as shown below:



Zechmeister and Toth³⁷ have isolated tri-, tetra- and possibly a hexasaccharide from the hydrolysis of cellulose with concentrated hydrochloric acid and advance this as evidence of a chain structure for cellulose. Haworth and his coworkers,^{40, 40A} have adduced evidence of a straight chain formulation for cellulose on the results of partial and complete hydrolysis of methylated cellulose. By partial hydrolysis a trisaccharide and possibly a tetrasaccharide were obtained and, on complete hydrolysis, 0.6 per cent of 2:3:4:6-tetramethyl glucopyranose in addition to the main product—2:3:6-trimethyl glucose. On the basis of these results Haworth concludes that the cellulose molecule has a molecular weight of about 30,000 and consists of a uniform chain of β -glucose residues.

Against such evidence, Bell has shown that by the hydrolysis of methylated cellulose which had not been pretreated by acetylation no tetramethyl glucopyranose could be detected, whereas the same material which had been acetylated prior to methylation gave 0.5 per cent of tetramethyl glucopyranose (approximately the same proportion as found by Haworth). Furthermore, Hess and his collaborators have questioned the homogeneity of the trisaccharide obtained by Zechmeister, Willstätter and Bertrand. They showed that the molecular weight determinations vary with the concentration and further that the "trisaccharide" can be separated into two fractions—a cellulose hydrate and a cellotriose—and they conclude that this evidence eliminates the supposed trisaccharide as evidence of a straight chain formulation for cellulose.

On evidence identical with that adduced for starch, the glucose residues of the cellulose molecule have a pyranoid ring structure. On purely physical grounds, Sponsler and Doré have interpreted their examination of the x-ray diagram of the crystal lattice of cellulose to show that the glucose residues in cellulose could only possess a pyranoid structure.

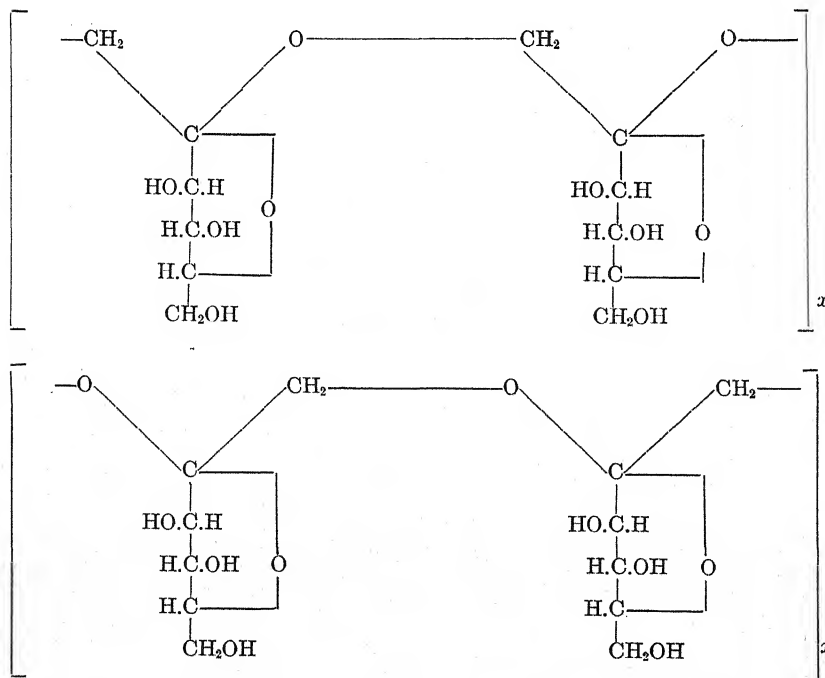
Since starch is composed of α -glucosidic residues and cellulose of β -glucosidic residues, by analogy it might be expected that a com-

parison of the optical rotations would show a generic relationship, and the following figures are of interest:

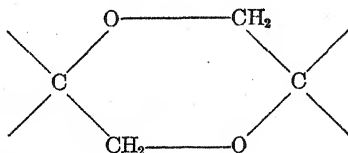
| | $[\alpha]_D$ | | $[\alpha]_D$ |
|---|--------------|--------------------------|--------------|
| α -Methylglucoside..... | +159 | Triacetyl starch..... | +170 |
| Tetra-acetyl- α -methylglucoside.. | +131 | Trimethyl starch..... | +208 |
| Tetramethyl- α -methylglucoside.. | +154 | | |
| β -Methylglucoside..... | - 34 | Triacetyl cellulose..... | - 22 |
| Tetramethyl- β -methylglucoside.. | - 17 | Trimethyl cellulose..... | - 18 |
| Tetra-acetyl- β -methylglucoside.. | - 18 | | |

Inulin.—By the hydrolysis of inulin with dilute acids, crystalline fructopyranose is obtained. By hydrolysis of trimethyl inulin, however, the main fraction (about 90 per cent) is obtained as 3:4:6-trimethyl fructofuranose, thus giving absolute evidence that the essential structure of the inulin molecule is based on fructofuranose units. Both Irvine⁴⁴ and Haworth⁴⁵ have isolated a small quantity of 1:3:4:6-tetramethyl fructofuranose from the cleavage products of trimethyl inulin and suggest on this basis that the inulin molecule consists of a chain of 30 fructofuranose units having a molecular weight of 5000.

On this evidence two arrangements are possible and as there is no evidence at present to distinguish between the α or the β configuration, both schemes are given in the following figures:



Against such a homogeneous structure there is the fact that a portion of the inulin molecule resists hydrolysis. Jackson and McDonald have isolated three nonreducing difructose anhydrides which would appear to indicate that inulin does not consist of a homogeneous molecule. Recently, however, it has been shown that on acetylation followed by methylation and hydrolysis, the difructose anhydrides give 3: 4: 6-trimethyl fructofuranose, and Haworth considers that the stability of these anhydrides is due to their possessing a dioxane type of linkage; *viz.*:



Furthermore, Haworth considers that these anhydrides are purely secondary products of the hydrolytic cleavage of inulin. Fructose is well known to be very unstable toward acids, especially in the furanoid form, and readily forms derivatives of furfural as is evidenced by the isolation of ω -methoxy-5-methylfurfural by Irvine from the products of hydrolysis of trimethyl inulin.

For lack of definite evidence, the homogeneity of the inulin molecule should be considered with reservations.

Hibbert, Tipson and Brauns¹⁴ have examined the synthetic polysaccharide, levan, formed by the action of *B. mesentericus* and *B. subtilis* on sucrose. This polysaccharide gives a triacetate differing markedly from that of inulin. It also gives a trimethyl derivative which yields 1: 3: 4-trimethyl fructofuranose on hydrolysis.

Glycogen.—Glycogen closely resembles starch, being a polysaccharide of formula $(C_6H_{10}O_5)_n$ in which each C_6 unit has three free hydroxyl groups. On methylation glycogen gives a trimethyl derivative which has the same composition as trimethyl starch and is chemically indistinguishable from it; likewise they have identical optical rotation, $[\alpha]_D + 208^\circ$ (in chloroform). Hydrolysis of trimethyl glycogen yields 2: 3: 6-trimethyl glucose and a small quantity of tetramethyl glucopyranose. It would therefore appear to be composed of α -glucosidic residues like starch. The difference between starch and glycogen presumably lies in the number of glucose residues in the chain composing the molecule. On the basis of the amount of tetramethyl glucose isolated, Haworth and Percival⁴² consider glycogen to contain a chain of twelve glucose residues in its molecule. There is no evidence in favor of constituting the glycogen molecule on a furanose basis.

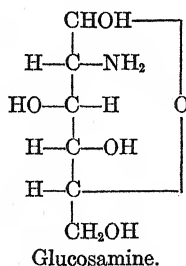
Haworth has isolated di-, tri- and tetrasaccharides similar to those obtained from starch, by degradation of trimethyl glycogen with acetyl bromide.

Thus, the weight of evidence favors the glycogen molecule as a chain of twelve α -glucose residues, but final confirmation of such a structure is dependent, as in the case of starch, on the proof that partial hydrolysis of the molecule has not taken place prior to the final hydrolysis of the methylated glycogen.

Xylan.—Xylan is a pentose polysaccharide and appears to be built upon the model of starch and cellulose. On methylation, it forms a dimethyl xylan, which on hydrolysis gives 2:3-dimethyl xylose.

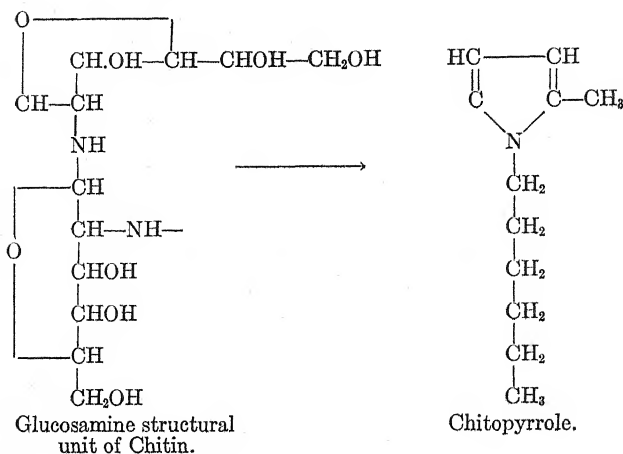
It may be pointed out here that the hemicelluloses which occur in wood consist of glucose and glucuronic acid residues. Carbon dioxide may be eliminated from such hemicelluloses and by some such mechanism the pentosans may be formed in nature from the hexose polysaccharides.

Chitin.—Chitin forms the main skeletal material of the *Insecta* and *Crustacea* in the animal kingdom and it is found as skeletal tissue in the fungi. So far no essential differences have been found in the chitin from vegetable or animal sources. It has an elementary composition of $C_{32}H_{54}O_{21}N_4$. By hydrolysis with concentrated acid, it yields glucosamine and acetic acid in proportions which indicate that there is an acetyl group for each glucosamine residue. By less drastic hydrolysis, the *N*-acetyl derivative of glucosamine is obtained. When chitin is fused with alkalis it breaks up into two molecules of acetic acid and chitosan, which has the composition $C_{28}H_{50}O_{19}N_4$. Neither chitin nor chitosan reduces Fehling's solution and hence the reducing groups of the glucosamine residues are concerned in linkages between the various units of the molecule.

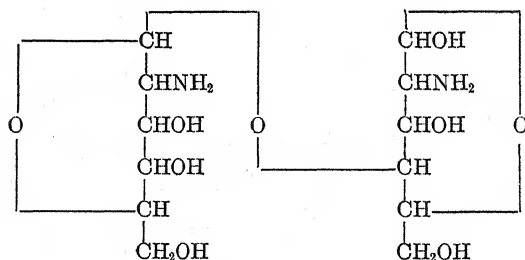


The further question, as to whether one is dealing here with a purely glycosidic linkage between the reducing group of one glucosamine residue and a hydroxyl group of a second (as in other polysaccharides), or with a linkage to the amino group of the second glucosamine residue has provoked much controversy.

Karrer has upheld the second view since he obtained chitopyrrole in a yield of 1 per cent by distilling chitin over zinc. The transformation of chitin free from acetic acid into chitopyrrole is considered to take place in the following manner:

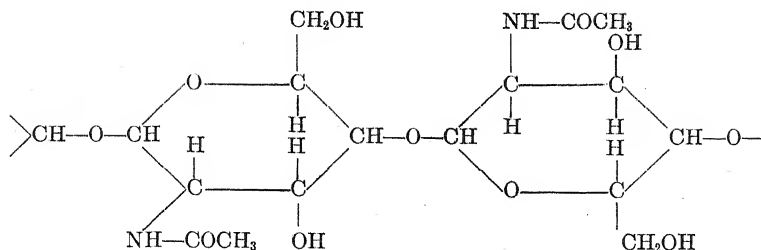


According to this scheme, Karrer considers chitin to have an aldehyde-ammonia linkage rather than that of a true complex carbohydrate. Bergmann, Zervas and Silberkweit have obtained a disaccharide, by the acid hydrolysis of chitin, which gave an octa-acetate. Two of the acetyl groups were more resistant to hydrolysis than the others and these authors consider that they are attached to the nitrogen atoms and they further conclude that the nitrogen atoms do not take part in the glycosidic union between the two components:



Since chitobionic acid on acetylation gives a 2:3 unsaturated acid whereas glucosaminic acid on similar treatment gives a doubly unsaturated acid, Bergmann and his coworkers have suggested that the components of the disaccharide are united at positions 1 and 4, as indicated in the above formula. Zechmeister obtained, by the hydrolysis of chitin with cold hydrochloric acid followed by acetylation, *N*-acetyl glucosamine and chitotriose undeca-acetate, thus supporting the conclusions of Bergmann regarding the structure of chitin.

On the basis of an *x*-ray examination of the crystal-lattice of chitin Meyer and Mark have suggested that the glucosamine residues are mutually united by <1,4> linkages alternately turned through 180° so that they appear as in the following formula, thus supporting the older formation proposed by Irvine and others:



Mucins and Mucoids.⁴⁵—The mucoproteins are widely distributed in nature and in reality conjugated proteins, *i. e.*, they contain a prosthetic group which is carbohydrate in character. This carbohydrate group is composed of four components in equimolecular proportions, *viz.*, sulfuric acid, acetic acid, a hexosamine and glucuronic acid.

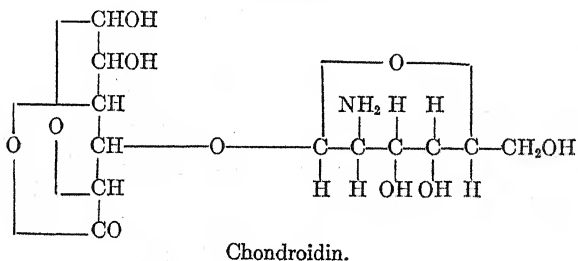
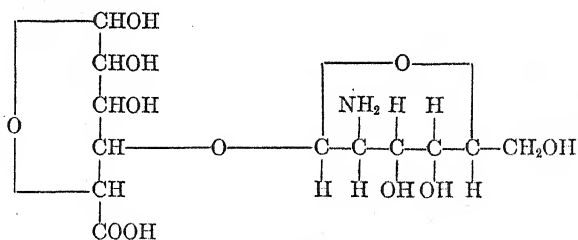
Levene and La Forge⁴⁶ isolated an aminohexose from the conjugated sulfuric acid of nasal septa which they named "chondrosamine." Eventually, Levene⁴⁷ brought forward evidence to show that chondrosamine was 2-aminogalactose.

The work of Levene and López-Suárez⁴⁸ showed that the aminohexose of the conjugated sulfuric acid of the mucin of pigs' stomachs differed from that previously isolated from chondroitin sulfuric acid in so far as the aminohexose was 2-aminoglucose. On this basis it is possible to divide the conjugated sulfuric acids into two groups: Group I contains those mucoproteins yielding chondroitin sulfuric acid, which contains chondrosamine as a constituent part of its molecule. They occur in cartilage, tendons, aorta and sclera. Group II contains those mucoproteins yielding mucoitin sulfuric acid, which contains glucosamine as a constituent part of its molecule. They occur in the navel cord, vitreous humor, cornea, gastric mucus and in serum mucoid. This latter group may be further subdivided into two groups depending on distinct differences in their physical properties. The mucoitin sulfuric acid of funis mucin is characterized by its greater insolubility and by more distinct colloidal properties whereas the mucoitin sulfuric acid of gastric mucosa is more soluble in water and is precipitated from an aqueous solution only by an excess of glacial acetic acid.

The mucoitin sulfuric acid is much less stable than chondroitin sulfuric acid and it is apt to undergo partial degradation during isolation; also, it is difficult completely to free the acids of group II from the adhering protein material.

Chondroitin Sulfuric Acid.—Chondroitin sulfuric acid is an amorphous substance soluble in water but insoluble in organic solvents, and with no action on Fehling's solution without preliminary hydrolysis. It does not contain a free primary amino group and acts as a dibasic acid. Sulfuric acid, in ester linkage, retains only one ionizable hydrogen atom and the carboxyl group of the glucuronic acid residue contributes the second, to give the molecule its dibasic properties. Thus the glucuronic acid component cannot be joined to the remainder of

the molecule through the carboxyl group. By the elimination of the sulfuric acid residues *chondroitin* is formed and this substance does not reduce Fehling's solution nor does it contain a free primary amino group. By degradation of chondroitin sulfuric acid with strong hydrochloric acid, a reducing disaccharide, chondrosin, is formed. Levene and La Forge⁴⁹ have shown this to contain both a free primary amino group and a carboxyl group. By oxidation of this disaccharide prior to hydrolysis, saccharic acid was obtained among the hydrolytic products, thus demonstrating that the reducing group of the glucuronic acid portion of the molecule was free. This result was confirmed by the isolation of chondroidin (by treatment of chondrosin with oxalic acid) which was shown by Levene and López-Suárez⁵⁰ to be a crystalline anhydride of chondrosin. These two components have the following type of structure:

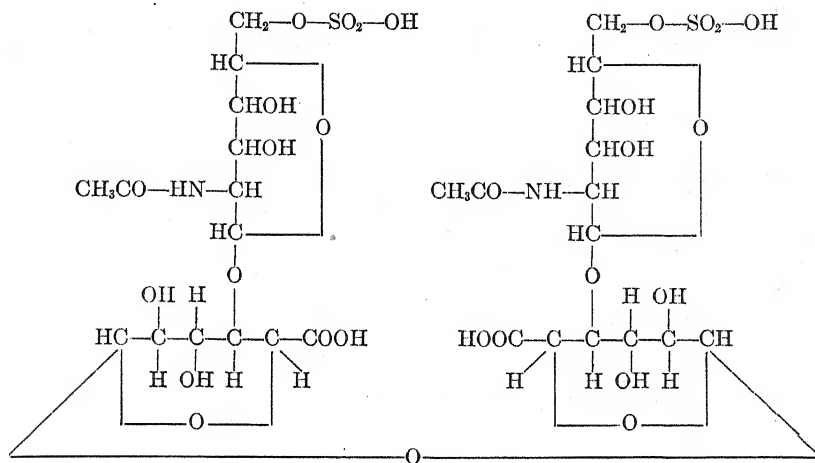


Thus there is only one detail of the structure of chondrosin which is left in doubt—the point of union of the two residues. Since the reducing group of the uronic acid is free and positions 3 and 5 in this residue are occupied in chondroidin by the lactal and lactone rings respectively, the aminohexose must be joined to the glucuronic acid through either position 2 or 4. Since there is no experimental evidence at present on this point, a union at carbon atom 4 has been tentatively suggested by analogy with other disaccharide linkages.

Structure of Chondroitin Sulfuric Acid.—Since the sulfuric acid-free chondroitin does not contain a free primary amino group it is evident that the sulfuric acid residue of the parent substance is not attached to the amino group. By purely circumstantial evidence it has been shown that the acetyl residue is condensed with the amino group.

The fact that chondroitin sulfuric acid does not reduce Fehling's

solution suggests that the reducing group of the glucuronic acid and the amino group of the chondrosamine unit are connected by ring formation. Levene and La Forge, however, have offered a second and more satisfactory explanation to account for the nonreducing behavior of chondroitin sulfuric acid. They suggest that two molecules of chondrosin are joined through their reducing groups in a manner similar to that in the nonreducing disaccharides, as follows:



In a private communication, Levene suggests that chondroitin sulfuric acid may have a chain structure similar to that of the other polysaccharides, each unit being a chondroitin sulfuric acid containing the four components.

The trehalose-like structure formulated previously by Levene and La Forge was suggested arbitrarily to express the nonreducing properties of the substance.

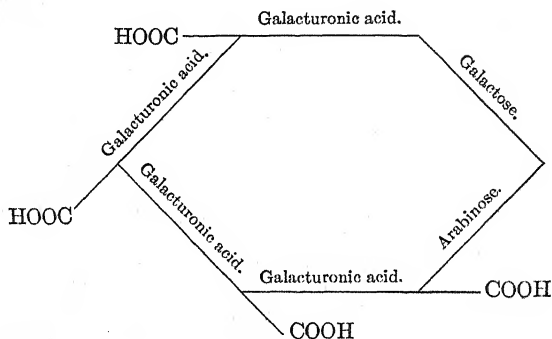
Mucoitin Sulfuric Acid.—Levene and López-Suárez have demonstrated that the sulfuric ester residue within mucoitin sulfuric acid is much less stable than in chondroitin sulfuric acid and for this reason is considered to occupy different positions in the two molecules. By hydrolysis of mucoitin sulfuric acid with mineral acids a disaccharide, mucosin, is obtained which is similar in all respects to chondrosin with the exception that the aminohexose is glucosamine instead of chondrosamine. It is considered to be the key to the structure of mucoitin sulfuric acid.

The Pectins.—The pectins belong to the vegetable mucilages, which are carbohydrate in character and are widely distributed in nature. The pectins of plants occur in a form insoluble in cold water and Ehrlich considers that all chemical processes which lead to a soluble form of natural pectin are in reality chemical degradations. The pectin of the beet-root consists of two components which may be separated by extraction with 70 per cent alcohol. The soluble material (30 per

cent) is an araban which may be hydrolyzed to *l*-arabinose and is a true polysaccharide. The second component (70 per cent) is a calcium-magnesium salt of the methyl ester of pectic acid. These two components are considered to be combined in chemical combination in the original pectin. The free pectic acid contains the following components: Methyl alcohol, acetic acid, arabinose, galactose and galacturonic acid.

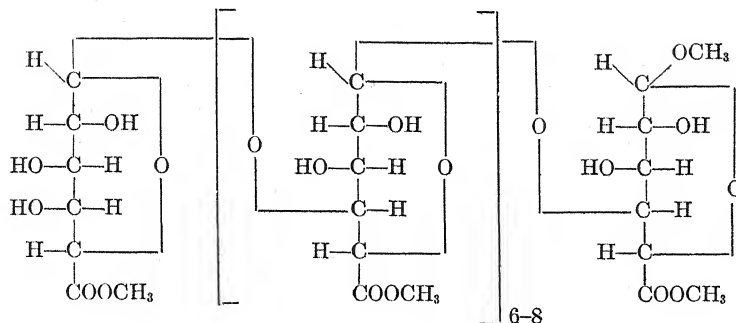
On the basis of the amounts of the various constituent units in pectic acid it has been assigned the structure of a triacetyl-arabino-galacto-dimethoxytetragalacturonic acid. The points of attachment of the acetyl groups have not been deduced but the two methoxyl groups are attached to two of the four carboxyl groups as ester residues.

Ling, Nanji and Paton consider beet pectic acid to be a trimethyl ester of the same complex acid and they have proposed the following skeleton structural diagram for pectic acid:



In 1929 Ehrlich isolated a tetragalacturonic acid which he claimed was the basal unit of the pectin molecule. In 1932 he described two forms of the tetragalacturonic acid: One, a 4-membered ring structure and the second, an open chain form derived from the former by the opening of one of the saccharide linkings. Ehrlich considers the 4-membered ring structure as the nucleus to which all the other components of the pectin molecule are attached.

Link and his coworkers⁵¹ have isolated a portion of the pectin molecule which is resistant to the hydrolytic action of methyl alcohol containing dry hydrogen chloride. During the course of the reaction the carboxyl groups become esterified. On deesterification of this resistant portion, the product retains a small amount of methoxyl residue which can only be in glycosidic union with a terminal galacturonic acid residue. On the basis of the percentage of methoxyl in the molecule, Link suggests that the resistant portion is a chain of 8-10 galacturonic acid residues as shown in the following figure and also that the basal unit of the pectin molecule contains at least 8-10 molecules of galacturonic acid.



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REFERENCES

1. Tollens, B.: *Ber.*, **16**, 921 (1883).
2. Lowry, T. M.: *J. Chem. Soc.*, **75**, 211 (1899); *Z. physik. Chem.*, **130**, 125 (1927).
3. Tanret, C.: *Bull. soc. chim.*, **15**, 195, 349 (1896); **17**, 802 (1897).
4. Fischer, E.: *Ber.*, **26**, 2400 (1893).
5. Fischer, E.: *Ber.*, **28**, 1145 (1895).
6. Kiliani, H.: *Ber.*, **18**, 3066 (1885); **19**, 221, 767, 1128 (1886).
7. Nef, J. U.: *Ann.*, **403**, 204 (1914).
8. Haworth, W. N., and Nicholson, V. S.: *J. Chem. Soc.*, 1899 (1926).
9. Goodyear, E. H., and Haworth, W. N.: *J. Chem. Soc.*, 3136 (1927); Haworth, W. N., *The Constitution of the Sugars* (1929).
10. Fischer, E.: *Ber.*, **47**, 1980 (1914); Haworth, W. N., Hirst, E. L., and Webb, J. I.: *J. Chem. Soc.*, 651 (1930).
11. Hudson, C. S.: *J. Am. Chem. Soc.*, **31**, 66 (1909).
12. Purdie, T., and Irvine, J. C.: *J. Chem. Soc.*, **83**, 1021 (1903); **85**, 1049 (1904); Irvine, J. C., and Cameron, A.: *J. Chem. Soc.*, **87**, 900 (1905).
13. Haworth, W. N.: *J. Chem. Soc.*, **107**, 8 (1915).
14. Hibbert, H., Tipson, R. S., and Brauns, F.: *Can. J. Res.*, **4**, 221 (1931).
15. Haworth, W. N., Hirst, E. L., and Miller, E. J.: *J. Chem. Soc.*, 2436 (1927).
16. Levene, P. A., and Tipson, R. S.: *Science*, **74**, 521 (1931); *J. Biol. Chem.*, **94**, 809 (1932); **97**, 491 (1932); **101**, 529 (1933).
17. Levene, P. A., and Meyer, G. M.: *J. Biol. Chem.*, **54**, 805 (1922); **57**, 317 (1923); **60**, 173 (1924).
18. Levene, P. A., and La Forge, F. B.: *J. Biol. Chem.*, **15**, 69, 155 (1913).
19. Ledderhose, G.: *Z. physiol. Chem.*, **2**, 213 (1878); **4**, 139 (1880); Neuberg, C.: *Biochem. Z.*, **43**, 500 (1912).
20. Nef, J. U.: *Ann.*, **357**, 214 (1907); **376**, 1 (1910).
21. Haworth, W. N., and Peat, S.: *J. Chem. Soc.*, 3094 (1926).
22. Zemplén, G.: *Ber.*, **59**, 1254 (1926).
23. Levene, P. A., and Simms, H. S.: *J. Biol. Chem.*, **65**, 31 (1925).
24. Purdie, T., and Paul, D. M.: *J. Chem. Soc.*, **91**, 289 (1907).
25. Fischer, E.: *Ber.*, **47**, 1980 (1914).
26. Irvine, J. C., Fyfe, A. W., and Hogg, T. P.: *J. Chem. Soc.*, **107**, 524 (1915).
27. Haworth, W. N.: *J. Chem. Soc.*, **117**, 199 (1920).
28. Pictet, A.: *Rev. gén. sci.*, **35**, 668 (1924).
29. Fischer, E.: *Ber.*, **23**, 3687 (1890).
30. Fischer, E., and Delbrück, K.: *Ber.*, **42**, 2776 (1909).
31. Helferich, B.: *Z. angew. Chem.*, **41**, 871 (1928).
32. Freudenberg, K., Noë, A., and Knopf, E.: *Ber.*, **60**, 238 (1927).
33. Pictet, A., and Vogel, H.: *Helv. chim. Acta*, **11**, 436 (1928).
34. Zemplén, G., and Gerecs, A.: *Ber.*, **62**, 984 (1929); Irvine, J. C., Oldham, J. W. H., and Skinner, A. F.: *J. Am. Chem. Soc.*, **51**, 1279 (1929); Irvine, J. C., and Stiller, E. T.: *J. Am. Chem. Soc.*, **54**, 1079 (1932).

35. Hess, K., and Trigus, C.: *Ber.*, **61**, 1932 (1928); Pringsheim, H., and Eissler, F.: *Ber.*, **46**, 2959 (1913); Pringsheim, H.: *Ber.*, **59**, 3008 (1926).
36. Pictet, A., and Sarasin, J.: *Helv. chim. Acta*, **1**, 87 (1918).
37. Zechmeister, L., and Tóth, G.: *Ber.*, **64**, 854 (1931).
38. Freudenberg, K., Kuhn, W., Dürr, W., Bolz, F., and Steinbrunn, G.: *Ber.*, **63**, 1510 (1930).
39. Haworth, W. N., and Percival, E. G. V.: *J. Chem. Soc.*, 1342 (1931).
40. Haworth, W. N., Hirst, E. L., and Thomas, H. A.: *J. Chem. Soc.*, 824 (1931).
- 40A. Haworth, W. N., and Machemer, H.: *J. Chem. Soc.*, 2270 (1932).
41. Hirst, E. L., Plant, M. M. T., and Wilkinson, M. D.: *J. Chem. Soc.*, 2375 (1932).
42. Haworth, W. N., and Percival, E. G. V.: *J. Chem. Soc.*, 2277 (1932).
43. Haworth, W. N., Hirst, E. L., and Percival, E. G. V.: *J. Chem. Soc.*, 2384 (1932).
44. Irvine, J. C.: *Chemistry Industry*, **51**, 263 (1932); *Nature*, **129**, 470 (1932).
45. Levene, P. A.: *Hexosamines and Mucoproteins* (1925).
46. Levene, P. A., and La Forge, F. B.: *J. Biol. Chem.*, **18**, 123 (1914).
47. Levene, P. A.: *J. Biol. Chem.*, **31**, 609 (1917).
48. Levene, P. A., and López-Suárez, J.: *J. Biol. Chem.*, **25**, 511 (1916).
49. Levene, P. A., and La Forge, F. B.: *J. Biol. Chem.*, **15**, 69 (1913).
50. Levene, P. A., and López-Suárez, J.: *J. Biol. Chem.*, **45**, 467 (1921).
51. Morell, S., Baur, L., and Link, K. P.: *J. Biol. Chem.*, **105**, 1 (1934).
52. Szent-Györgi, A.: *Biochem. J.*, **22**, 1387 (1928).
53. Hirst, E. L., *et al.*: *J. Chem. Soc.*, 1270 (1933).
54. Reichstein, T., Grüssner, A., and Oppenauer, R.: *Nature*, **131**, 280 (1933); *Helv. chim. Acta*, **16**, 1019 (1933); Haworth, W. N., Hirst, E. L., *et al.*: *J. Chem. Soc.*, 1419 (1933).
55. Harden, A., and Young, W. J.: *Proc. Chem. Soc.*, **21**, 189 (1905).
56. Morgan, W. T. J., and Robison, R.: *Biochem. J.*, **22**, 1270 (1928).
57. Neuberg, C.: *Biochem. Z.*, **88**, 432 (1918).
58. Harden, A., and Robison, R.: *Proc. Chem. Soc.*, **30**, 16 (1914); Robison, R.: *Biochem. J.*, **16**, 809 (1922).
59. Robison, R., and King, E. J.: *Biochem. J.*, **25**, 323 (1931).
60. Wróblewski, A.: *J. prakt. Chem.*, **64**, 1 (1901).
61. Pasteur, L.: *Ann. chim. phys.* (3), **58**, 323 (1860).
62. Neuberg, C., and Reinfurth, E.: *Biochem. Z.*, **89**, 365 (1918); **92**, 234 (1918); *Ber.*, **52**, 1677 (1919); **53**, 462 (1920); **53**, 1039 (1920); *Biochem. Z.*, **106**, 281 (1920); Connstein, W., and Lüdecke, K.: *Ber.*, **52**, 1385 (1919).
63. Neuberg, C., and Hirsch, J.: *Biochem. Z.*, **96**, 175 (1919); **100**, 304 (1919); Neuberg, C., Hirsch, J., and Reinfurth, E.: *Biochem. Z.*, **105**, 307 (1920); Neuberg, C., and Ursum, W.: *Biochem. Z.*, **110**, 193 (1920).

CHAPTER III

THE NEUTRAL FATS AND RELATED SUBSTANCES

THE substances to be considered in this chapter usually are called *fats*, *oils*, *lipoids*, *lipids*, or *lipins*. Unfortunately there is little uniformity in the definition and usage of these terms.

Fat.—As a rule, in organic chemistry, the word "fat" is applied only to triglycerides of fatty acids. However, it is also used widely as an inclusive term for all substances of a fatlike nature. To avoid confusion, it is customary to employ the term "neutral (or true) fat" when speaking specifically of fatty acid triglycerides.

Oil.—The word "oil" is applied to a great many substances which may have no chemical relationship whatsoever. Originally it probably meant merely a somewhat viscous liquid. Oils of biological origin are of two types: Fatty oils and essential or volatile oils. The term "fatty oil," or simply "oil," is employed in the same sense as the term "fat" when the substance is a liquid at room temperature. However, there is no sharp differentiation in usage. A fatty extract is frequently called "fat" even though it may be liquid, while many oils, such as coconut or palm oil, are solid at the usual temperature of the temperate zone.

Lipoid.—The word "lipoid" is used by some authors as a general term to include the true fats and all substances related to them. Other writers employ it to designate all substances associated with the fats except the fats themselves; while some restrict its use to fatty compounds containing nitrogen.

Lipid.—To avoid the confusion resulting from these inconsistencies, the Committee on the Reform of the Nomenclature of Biological Chemistry of the Fourth International Congress of Pure and Applied Chemistry resolved¹ that the word "lipoid" be dropped and that in its place the word "lipide" be used to "designate the group of substances which comprises the fat bodies and esters which possess analogous properties." (The cerebrosides, which contain no ester linkage, are not included under this definition.) Following this report, most American biochemists have adopted "lipide" or "lipid" as an inclusive term for the fats and fatlike substances. Unfortunately chemists have not agreed unanimously to this change. At the Ninth International Union of Pure and Applied Chemistry the National Committee of Great Britain recommended² that the term "lipide" be restricted to those compounds which yield by hydrolysis an alcohol or a sugar, one or several molecules of a base, and a fatty acid. This definition does not include the neutral fats, fatty acids, or the sterols.

Lipin.—The term "lipin" was proposed by Rosenbloom and Gies³ as an inclusive term for fats and associated substances. It has been

used to some extent in this sense. On the other hand MacLean⁴ proposed that its meaning be restricted to substances of a fatlike nature containing nitrogen; and some writers, especially British, have adopted this definition. The proposal of the National Committee of Great Britain, referred to above, amounts to a substitution of "lipide" for "lipin" as defined by MacLean.

It is evident from the foregoing discussion that there is no uniformity in the nomenclature of the fatty substances. Four different terms (fat, lipid, lipin, and lipin) are used to designate the total content of fatty substances in biological material, but any of these names may be used in a restricted sense. The student must interpret and compare experimental data on the basis of methods employed without regard to nomenclature.

The confusion is increased by differences among writers in defining the total fatty content of biological material. It is general biochemical practice to call by one of the above names the fraction obtained by extraction with one or more of the so-called "fat solvents." This is a matter of practical necessity since in most investigations it is impossible, for want of methods or time, to separate and isolate quantitatively the various compounds which are present. The solvents employed (alcohol, ether, petroleum ether, chloroform, benzene, acetone, etc.) differ widely in their solvent action and none of them is specific for the fatty compounds. Alcohol, for example, is a good solvent for many biological substances which are in no way related to the fats, while petroleum ether is a much more specific solvent but does not dissolve certain members of the fatty group (cerebrosides, etc.). Obviously, extracts made with different solvents may be quite different in amount and composition. Furthermore, all such extracts, regardless of solvent, contain substances, such as sterols and higher alcohols, which are not related to the fats chemically, and also more or less material of unknown composition.

While it is customary in practical work to define the fatty substances on the basis of solubility, they are more usually classified and defined in textbooks strictly according to chemical structure. It is evident that these two definitions are by no means equivalent. In a chapter on organic chemistry we must of necessity limit the discussion to substances of known structure. The following classes of compounds are generally included in the fatty group: Fatty acids and soaps, neutral fats, waxes, sterols, phosphatides, and cerebrosides. Fatty acids will be discussed first since they may be considered to be the building stones of the fatty substances.

FATTY ACIDS

Definition and Classification.—It is difficult to give an exact definition of a fatty acid. Some organic chemists limit the term to saturated monobasic acids of the aliphatic (open-chain) homologous series having the formula $C_nH_{2n}O_2$. Others include also unsaturated, aliph-

FATTY ACIDS OCCURRING IN NATURE

1. Saturated normal (straight-chain) acids having the empirical formula $C_nH_{2n}O_2$, or $C_{n-1}H_{2n-1}COOH$, or (with 3 or more carbon atoms) $CH_3(CH_2)_xCOOH$.

| Name. | Formula. | Name. | Formula. |
|---------------|-------------------|---------------------|-------------------|
| Acetic..... | $C_2H_4O_2$ | Palmitic..... | $C_{16}H_{32}O_2$ |
| Butyric..... | $C_4H_8O_2$ | Stearic..... | $C_{18}H_{36}O_2$ |
| Caproic..... | $C_6H_{12}O_2$ | Arachidic..... | $C_{20}H_{40}O_2$ |
| Caprylic..... | $C_8H_{16}O_2$ | Behenic..... | $C_{22}H_{44}O_2$ |
| Capric..... | $C_{10}H_{20}O_2$ | Lignoceric (?)..... | $C_{24}H_{48}O_2$ |
| Lauric..... | $C_{12}H_{24}O_2$ | Cerotic..... | $C_{26}H_{52}O_2$ |
| Myristic..... | $C_{14}H_{28}O_2$ | Melissic..... | $C_{30}H_{60}O_2$ |

2. Unsaturated acids having the empirical formula $C_nH_{2n-2u}O_2$ where "u" = number of double bonds.

| Name. | Formula. | Number of double bonds. |
|--------------------------|-------------------|-------------------------|
| Oleic..... | $C_{18}H_{34}O_2$ | 1 |
| Erucic..... | $C_{22}H_{42}O_2$ | 1 |
| Linoleic or linolic..... | $C_{18}H_{32}O_2$ | 2 |
| Eleostearic..... | $C_{18}H_{30}O_2$ | 2 |
| Linolenic..... | $C_{18}H_{30}O_2$ | 3 |
| Arachidonic..... | $C_{20}H_{32}O_2$ | 4 |
| Clupanodonic..... | $C_{22}H_{34}O_2$ | 5 |

3. Hydroxy acids.

| | | |
|-----------------|-------------------|---|
| Ricinoleic..... | $C_{18}H_{34}O_3$ | 1 |
|-----------------|-------------------|---|

atic monobasic acids. In either case the classification is made on the basis of chemical structure without regard to occurrence or nonoccurrence in nature. Biochemists on the other hand consider any organic acids which occur in neutral fats or fatty substances to be fatty acids. For example, cyclic acids such as chaulmoogric acid are called fatty acids when they occur naturally as constituents of fats.

Most of the fatty acids found in nature are monobasic, normal (straight-chain) compounds with an *even* number of carbon atoms. Straight-chain acids with odd numbers of carbon atoms and isomeric branched-chain acids with both even and odd numbers of carbon atoms are well known in the laboratory, but it is questionable whether they occur naturally in appreciable quantities. Nevertheless they are usually classed with the fatty acids because of their similarity in structure and properties.

Anderson⁵ has isolated saturated *liquid* acids from tubercle bacilli and other organisms. The structure of these interesting substances has not yet been established but it is evident that they must have a branched-chain structure since they are optically active and differ markedly in properties from straight-chain acids of the same elementary composition.

All even-numbered members of the saturated series from C_2 up to C_{30} have been found in biological material, but unsaturated acids with less than eighteen carbons occur only rarely. (Several isomeric C_{16} acids with one double bond have been isolated but they are of little quantitative importance. Recently Bosworth and Brown⁶ have demonstrated the presence of unsaturated C_{10} (decenoic) and C_{14} (tetradecenoic) acids in butter.) From the quantitative point of view, the most important fatty acids are palmitic, stearic and oleic which are found in all animal and most plant fats and oils.

Physical Properties.—Fatty acids of the saturated series with ten or fewer carbon atoms are known as the *volatile* fatty acids, since they may be distilled readily with steam.

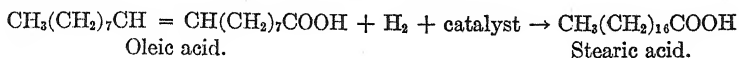
Saturated fatty acids with ten or more carbon atoms have melting points above ordinary room temperature. On the other hand, most of the unsaturated acids listed in the table on page 111 are liquid, even though their molecular weights are high; but fatty acids are not necessarily liquid because they contain one or more double bonds. The melting point is more a function of the spatial arrangement of the molecule. For example, oleic acid is liquid while its isomer elaidic acid melts at 45° C. Sorbic acid is a high-melting solid; although it is highly unsaturated and contains only six carbon atoms.

As is to be expected, solubility in water decreases with increasing length of chain. Up to and including caproic acid, the fatty acids are soluble (water dissolves approximately 0.9 per cent caproic acid at 15° C.), but lauric acid is only slightly soluble in boiling water and higher homologues are almost entirely insoluble. In general, the fatty acids dissolve readily in the "fat solvents," but those of high molecular weight are only sparingly soluble in *cold* alcohol.

Chemical Properties and Reactions.—The most important chemical property of the fatty acids is their acid nature. Their possession of an acid carboxyl group enables them to form salts with alkalis, esters with alcohols, and amides with amines. Salts of fatty acids of high molecular weight are known as soaps. The neutral fats, waxes, and phosphatides (except sphingomyelin) are esters, while the cerebrosides and sphingomyelin are amides of fatty acids. These compounds will be discussed in other sections of this chapter.

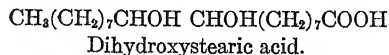
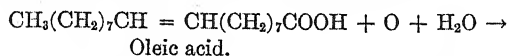
The saturated fatty acids possess only one polar group (the carboxyl) and are relatively inert. The unsaturated fatty acids are more reactive due to the presence of one or more double bonds. They are readily reduced or oxidized, take up iodine and bromine, and exhibit two types of isomerism.

The complete reduction of an unsaturated fatty acid yields the corresponding saturated acid. For example,

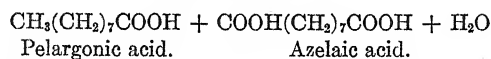
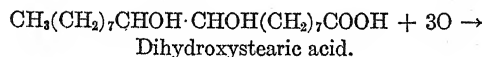


The reaction is carried out at an elevated temperature in an atmosphere of hydrogen and in the presence of a metallic catalyst.

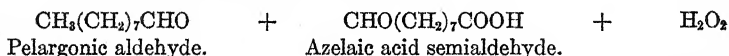
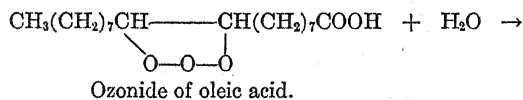
Mild oxidation of unsaturated acids with dilute potassium permanganate yields hydroxy acids. For example,



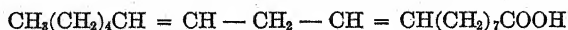
More vigorous oxidation of oleic acid splits the molecule at the double bond yielding two acids, one of which is monobasic and the other dibasic.



This reaction suggests that the double bond in oleic acid is in the center of the molecule, but it does not prove it since a shift in the position of the double bond may occur under the influence of the oxidizing agent. Confirmatory evidence is found in oxidation with ozone which unites at the double bond to form an ozonide. When the ozonide is heated with water, pelargonic aldehyde and the semialdehyde of azelaic acid are formed.

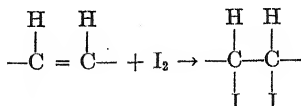


Mild oxidation of fatty acids with more than one double bond gives the corresponding hydroxy acids. Thus tetrahydroxystearic (sativic) acid and hexahydroxystearic (linusic) acid are formed from linolic and linolenic acids respectively. Stronger oxidation splits the molecule as in the case of oleic acid; but as several products are formed it is more difficult to draw conclusions concerning the position of the double bonds. Recent investigations⁷ make it fairly certain that linolic acid has the following structure:



Unsaturated fatty acids are oxidized spontaneously in the air.

Unsaturated fatty acids add halogens (iodine, bromine, etc.) at their double bonds.



This property is of great importance in the study of fats and related substances. The **iodine number** is the number of grams of halogen, calculated as iodine, which 100 Gm. of fatty acid (or other fatty substance) will take up. It is a measure of the degree of unsaturation and is used widely in studying changes of fats during metabolism, etc. It is usually determined by the Hübl, Wijs, Hanus, or Rosenmund-Kuhn-henn methods. In all of these procedures a weighed sample of the fatty substance under investigation is dissolved in chloroform or carbon tetrachloride, mixed with a measured quantity of a solution of halogenizing reagent and allowed to stand in a well-stoppered flask in the dark for twelve to eighteen hours (Hübl method), one-half hour (Wijs and Hanus methods), or fifteen minutes (Rosenmund-Kuhn-henn method).

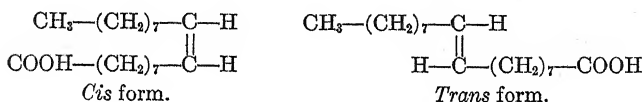
The halogenizing reagent in the Hübl method is iodo-mercuric chloride, which is made by mixing alcoholic solutions of iodine and mercuric chloride. The halogenizing reagents used in the Wijs and Hanus methods are ICl and IBr respectively. They are made by adding chlorine or bromine to a solution of iodine in glacial acetic acid. In the Rosenmund-Kuhn-henn method, pyridine sulfate dibromide is the active reagent. It is made by mixing solutions of pyridine, H_2SO_4 , and bromine in glacial acetic acid. In all of the methods, at the completion of the reaction, water and KI solution are added and the iodine, which is liberated by the excess reagent, is titrated with sodium thiosulfate. Blank determinations, in which no fatty substance is added, are carried through the procedure and titrated in the same way. The difference in the titration values of the blank and the unknown is equivalent to the halogen, expressed as iodine, which is absorbed by the sample.

Saturation with bromine is utilized in the separation of unsaturated fatty acids. The solubility of brominated fatty acids decreases as the bromine content increases. Hexa- and octabrom fatty acids (from fatty acids with three and four double bonds respectively) are insoluble in ether, while di- and tetrabrom fatty acids are soluble. Octabrom fatty acids are much more soluble in boiling benzene than hexabrom fatty acids. Tetrabrom fatty acids are insoluble in petroleum ether, while dibrom fatty acids are soluble. It is possible by proper application of these properties to determine the approximate amounts of fatty acids with one, two, three, or four double bonds present in a mixture.

Each of the **unsaturated fatty acids** may have a large number of **isomers**. One type of isomerism depends on the position of the double bonds in the molecule. There are sixteen positions (corresponding to sixteen isomers) in which the unsaturated group may be present in a straight-chain acid with the empirical formula of oleic acid. At least

four such isomers of oleic acid have been found in nature, but the exact position of the double bond has not been established with certainty in any of them. The number of possible isomers of fatty acids with more than one unsaturated group is large and several isomers of linolic acid are known. The position of the double bonds has not been determined.

Another type of isomerism depends on the spatial arrangement of the molecule. A fatty acid with one double bond may have either of two isomeric forms. For example, the treatment of oleic acid with nitrous acid changes it into another acid (elaidic) which differs markedly in physical properties. Elaidic acid melts at 45° C. while oleic acid melts at 13°–14° C. These two acids have the same empirical formula and are not isomers of the first type, since the double bond is in the same position in each. The following formulae represent their structure:



Hilditch⁸ has presented evidence which indicates strongly that oleic acid has the *cis* configuration. Elaidic acid has not been found in nature.

The number of possible stereomeric isomers equals 2_n , where "n" is the number of double bonds. Little is known concerning the isomers of fatty acids with more than one double bond. Eleostearic acid, which occurs naturally, is thought to be a spatial isomer of linolic acid.

Separation of Fatty Acids.—The volatile fatty acids are separated from nonvolatile fatty acids by steam distillation. The separation of saturated from unsaturated fatty acids is usually based on the fact that the lead salts of unsaturated fatty acids are much more soluble in alcohol or ether than those of the saturated fatty acids. However, lead salts of saturated fatty acids are not entirely insoluble, and so the separation is not complete.

The separation of the individual saturated fatty acids from each other is a difficult matter, since adjacent members of the series differ but little in physical properties. The separation may be accomplished by careful fractional distillation of the methyl esters of the fatty acids *in vacuo*.

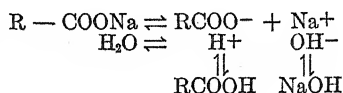
Hydroxy Fatty Acids.—Many hydroxy fatty acids of low molecular weight are known in nature. Some of these, such as lactic and β -hydroxybutyric acids, are of great importance, but since they differ markedly in their properties from the nonhydroxylated fatty acids and do not occur in fatty combinations (as neutral fats, phosphatides, etc.), they are not included with the true fatty acids. A few hydroxy fatty acids of high molecular weight occur in fatty combinations and are considered to be true fatty acids. Of these the only one of much quantitative significance is rincinoic acid, the glyceride of which is

the principal constituent of castor oil. It is a monohydroxy derivative of oleic acid with the following structure:



It is optically active since the carbon to which the hydroxyl group is attached is asymmetric. It also exhibits stereomeric isomerism due to the double bond and may be converted into an isomer, ricinelaidic acid, by nitrous acid just as oleic acid is converted to elaidic acid.

Soaps.—The soaps are salts of the nonvolatile fatty acids. Sodium and potassium soaps are somewhat soluble in water and comprise the ordinary soaps of commerce. In dilute solution soaps are partially hydrolyzed with the formation of alkali and free fatty acid:

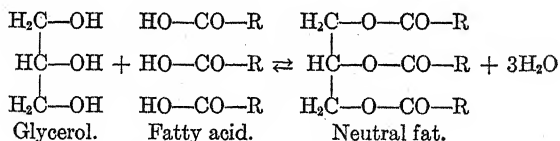


The free fatty acid which is formed probably unites with unhydrolyzed soap to form complex molecules.

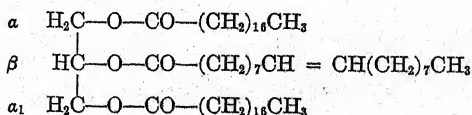
Alkaline earth and heavy metal salts of nonvolatile fatty acids are frequently referred to as soaps although they are insoluble in water and have none of the properties ordinarily associated with soap. Indeed, "hardness" of water is due to a high content of alkaline earths which precipitate the fatty acids of soap as insoluble salts.

NEUTRAL FATS AND OILS

Structure and Nomenclature.—The neutral or true fats (or simply fats) and fatty oils are esters formed by a combination of glycerol and fatty acids:



In the resulting fat or oil all three fatty acid radicles may be the same, as in the example shown, in which case it is called a *simple triglyceride*; or two or three different fatty acids may be present, in which case it is called a *mixed triglyceride*. Simple triglycerides are named according to their fatty acids. For instance, the triglyceride of stearic acid is tristearin or stearin; of oleic acid, triolein or olein, etc. The nomenclature of the mixed triglycerides depends on the position of the fatty acids. For example:



is β -Oleo- α - α_1 -distearin.

Physical Properties.—In general, the neutral fats and oils are similar in their physical properties to their constituent fatty acids. This is true especially when the fatty acids are of high molecular weight and consequently make up a very large part of the fat or oil molecule. In stearin, for example, the stearic acid radicle accounts for over 90 per cent of the molecular weight. The following table illustrates the similarity in melting points of simple triglycerides and their fatty acids

| Fatty acid. | Melting point. | Triglyceride. | Melting point. |
|---------------|-----------------------------|---------------|-----------------------------|
| Lauric..... | 43.6° | Laurin..... | 45°-46° |
| Myristic..... | 53.8° | Myristin..... | 55°-56° |
| Palmitic..... | 62.6° | Palmitin..... | 64°-65° |
| Stearic..... | 69.3° | Stearin..... | 71°-72° |
| Oleic..... | Liquid at room temperature. | Olein..... | Liquid at room temperature. |

The melting points of natural fats or oils, which are very largely composed of triglyceride mixtures, are fixed primarily by the proportionate amounts of different fatty acid radicles present. In most cases low-melting fats and oils contain a large proportion of liquid unsaturated fatty acids, of which oleic acid is by far the most important quantitatively.

Other factors are concerned to a lesser degree in establishing the melting point of a natural fat. Each constituent of a fatty mixture exercises an unpredictable influence on the melting points of the other constituents. The melting point of a mixture of two triglycerides may be below that of the lower melting or above that of the higher melting triglyceride. The melting points of mixed triglycerides vary to some extent with the position of the fatty acid radicles. Thus β palmito- α , α_1 dimyristin melts at 59.8-60° C. while α palmito- α , β dimyristin melts at 53°. ⁹

In general the neutral fats and oils are soluble in the same solvents as the fatty acids. However, in most solvents they are much less soluble than the fatty acids from which they are formed. This is to be expected, since the molecular weight of the fats is approximately three times that of their constituent fatty acids.

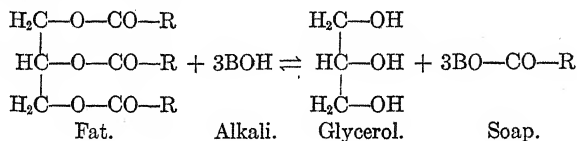
Chemical Properties and Reactions.—The most important chemical property of the neutral fats and oils is their capacity to be split by hydrolysis (saponification) into fatty acids and glycerol. The splitting may be accomplished by heating with water alone, by the action of catalysts such as acids, enzymes, etc., or by treatment with alkali. The equation for the hydrolytic reaction is the reverse of that given at the beginning of this section for the formation of the fats. Reaction with water does not take place at temperatures below 150° C., but at

high temperature and pressure it is fairly rapid. In the presence of acid, which acts as a catalyst, the reaction proceeds fairly rapidly at the boiling point of water at atmospheric pressure. The hydrolysis of fats is catalyzed also by enzymes called lipases which are widely distributed in nature. They are found in most, if not all, oily seeds, in the intestinal tract, and in many animal tissues.

One type of rancidity is due to the partial spontaneous hydrolysis of fats and oils with the production of odoriferous, volatile free fatty acids. Of course, only fats such as butter containing appreciable amounts of volatile fatty acid radicles are subject to this type of rancidity. Moisture, light and air are essential to the process. It is probable that traces of lipase catalyze the reaction.

In the laboratory and in industry the neutral fats and oils are usually split by treatment with alkali. This process is called **saponification** since soaps are formed in the reaction. However, the term "saponification" is used widely to designate any process of fat splitting, such as the hydrolyses already described, even though no alkali is used and no soap is formed.

The equation for the saponification of a representative fat follows:



Saponification with alkali in an aqueous medium is much faster than hydrolysis catalyzed by acid because the soap formed in the reaction helps to emulsify unsplit fat and bring it into more intimate contact with the alkali. In hot alcohol saponification is even more rapid since the fat and alkali are in homogeneous solution.

Fats and oils containing unsaturated fatty acid radicles may be reduced, oxidized, and saturated with halogen in the same manner as the free unsaturated fatty acids.

Partial catalytic hydrogenation of vegetable oils is used widely in industry to produce fats of higher melting point which are known as "lard substitutes." The reaction is carried out in an atmosphere of hydrogen at an elevated temperature in the presence of a metallic catalyst. A great many different catalysts such as iron, copper, vanadium, osmium, and beryllium have been proposed, but nickel has been most widely employed. It is used either as a finely divided powder, or it is deposited on inert substances such as carbon, kieselguhr, and silica in order to increase the surface. It has also been used in the form of salts or oxides. Increased pressure is usually employed in order to reduce the time of exposure of the fats to high temperatures, which tend to cause some decomposition and impairment of taste.

The **iodine number** of a fat is determined in the same way and has the same significance as the iodine number of a fatty acid (see p. 114).

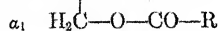
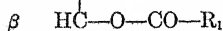
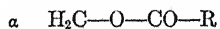
Mild **oxidation** of unsaturated fats and oils results in hydroxy derivatives; stronger oxidation splits the molecule at the double bonds without destroying the fatty acid-glyceryl linkages. The last reaction has been utilized by Hilditch in studying the composition of neutral fats.

Unsaturated fats and oils are susceptible of spontaneous autooxidation in the air in the presence of moisture and light. The phenomenon of *drying* is due primarily to such spontaneous oxidation. *Drying oils*, of which linseed oil is the best known example, form a tough, solid, highly insoluble substance on exposure to air. They are responsible for the drying of paint. The chemical changes which take place in drying are not known beyond the fact that oxygen is necessary for the process. In all probability polymerization also plays a rôle. Only highly unsaturated vegetable oils, containing a large proportion of fatty acids with three double bonds, possess the property of drying. Animal oils, though equally unsaturated, do not dry. The reason is not known. In general, the drying property of vegetable oils is proportional to their degree of unsaturation.

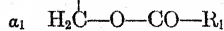
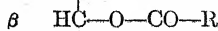
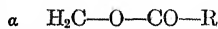
Spontaneous oxidation of unsaturated fats and oils is also responsible for a type of *rancidity* which is different from that caused by the liberation of volatile fatty acids by spontaneous hydrolysis. It results in the formation of odoriferous aldehydes, ketones, and fatty acids of low molecular weight. Fats such as butter, which contain large amounts of volatile and unsaturated fatty acid radicles, are susceptible to both types of rancidity, which may occur simultaneously.

Autoxidation of fats and oils is preceded by a period of inductance during which there is practically no absorption of oxygen. Recently Mattill¹⁰ has shown that several organic compounds have the property of greatly prolonging this induction period when added to fats and oils in very small amount (0.02 per cent). The most active compounds contained two phenolic hydroxyls in the ortho or para configuration. Substances such as these, which protect against autoxidation, when present in very low concentration, are called *antioxidants*. Mattill and coworkers have shown that several vegetable oils contain natural antioxidants. Mattill suggests that the presence of these substances accounts for the fact that vegetable fats and oils keep better than equally unsaturated animal fats and oils.

Two types of isomerism are possible in triglycerides. The first depends on the position of the fatty acid radicles. A triglyceride containing two radicles of one fatty acid and one of another may have either of two structures:

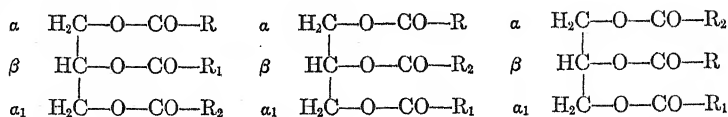


Symmetrical form.



Unsymmetrical form.

A triglyceride containing three different fatty acid radicles may have any one of three different structures:

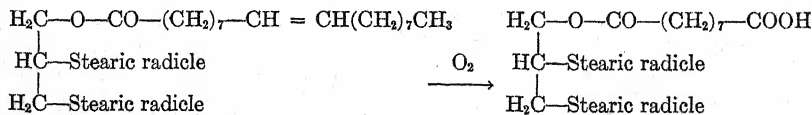


Such isomers may differ markedly in physical properties. For instance, α lauro- α_1, β -distearin melts at 50.6°C . while β lauro- α, α_1 -distearin melts at 59.8°C .¹¹

A second type of isomerism is possible in unsymmetrical triglycerides. In such compounds four different groups are attached to the center (β) carbon atom, which, therefore, is asymmetric. Optically active triglycerides have not been found in nature. Abderhalden and Eichwald¹² synthesized optically active mono- and di-butyryns.

Constitution of Natural Fats and Oils.—Until recently it was thought that natural fats and oils were composed principally of simple triglycerides. However, several investigators have attempted to isolate simple triglycerides from many natural fats and oils but have found only traces in most of the samples examined. For this reason it is now thought that fats and oils are mainly mixtures of mixed triglycerides.

The actual separation from natural fats and identification of pure mixed triglycerides by physical methods (fractional crystallization, etc.) is very difficult, since they differ but little in physical properties. In the past few years much new information concerning the composition of natural fats and oils has been presented by Hilditch and his coworkers. Hilditch and Lea¹³ devised an ingenious fractionation procedure based on the oxidation of fats and oils with KMnO_4 . Completely saturated triglycerides are unaffected by the treatment. Unsaturated triglycerides are oxidized and split at their double bonds, with the introduction of a carboxyl group into each unsaturated fatty acid radicle. For example, oleodistearin gives azelaodistearin:



The triglyceride acids resulting from oxidation are soluble in alkaline aqueous solution and may be readily separated from the insoluble saturated triglycerides. The proportion of completely saturated and partially unsaturated triglycerides has been determined by this method in a large number of natural fats and oils. By determining the proportionate amounts of different fatty acids present in the saturated triglycerides and comparing with the fatty acid make-up of the entire fat, it is possible to calculate the ratio of saturated to unsatu-

rated fatty acid molecules in the mixed saturated-unsaturated (plus tri-unsaturated) triglycerides of the original fat. The work done so far has led to the conclusion that vegetable seed fats and oils contain a negligible amount of fully saturated triglycerides until the molar proportion of saturated acids of the whole fat approaches 60 per cent. In other words, there appears to be a tendency toward an even distribution of fatty acids among the glycerides. In animal fats and vegetable mesocarp fats the structure is quite different. In these fats the molar proportion of fully saturated glycerides increases in a regular manner following corresponding increase in the proportion of saturated to unsaturated acids as a whole.¹⁴

King and coworkers¹¹ have recently attacked the problem of fat constitution by a different method. They have synthesized pure mixed triglycerides and determined their properties. Such data should be of great value in identifying triglycerides separated from natural fats and oils. King and coworkers point out that most of the methods which have been used for triglyceride synthesis give compounds in which the position of the fatty acid radicles is not certain, since shifting of position may occur at the elevated temperatures employed. They have employed the method of Fischer in which the condensation of fatty acid chloride with glycerol hydroxyl is carried out in cold quinoline or pyridine. Hydrochloric acid, formed in the reaction, is taken up by the basic solvent.

Waxes.—The term "wax" is applied to many natural products, of which beeswax is the best known, having properties similar to those of the fats. In organic chemistry it is applied specifically to natural products consisting principally of esters which may be thought of as fats in which glycerol is replaced by other alcohols. Most of these alcohols have a relatively high molecular weight and a single hydroxyl group. The best known are cetyl alcohol ($C_{16}H_{33}OH$), ceryl alcohol ($C_{26}H_{53}OH$), myricyl alcohol ($C_{30}H_{61}OH$), and the sterols. These alcohols form solid waxes with properties similar to those of the fats.

Liquid waxes have been found only in animal tissues. They probably represent esters of unsaturated alcohols and unsaturated fatty acids.

Recently Anderson and Newman¹⁵ reported that "the neutral fat from the human tubercle bacillus which is soluble in cold acetone is . . . not a glyceride but a complex ester of fatty acids with trehalose." In this connection the finding of Anderson that saturated, branched-chain acids occur in relatively large quantity in bacteria is of interest. One of these acids (phthioic acid from tubercle bacilli), "when injected intraperitoneally, stimulates the proliferation of monocytes, epithelioid cells, and giant cells, with the formation of tubercular tissue."⁵ Apparently, bacteria differ markedly from other organisms in their fatty metabolism.

Sterols.—The sterols are monohydroxy alcohols of high molecular weight. They are usually grouped with the fatty substances, first

because they occur as fatty acid esters (waxes), and secondly because their solubilities are similar to those of the fats. (See Chapter IV.)

FATTY SUBSTANCES CONTAINING BOTH PHOSPHORUS AND NITROGEN (PHOSPHATIDES)

Nomenclature.—The terms “phosphatide,” “lecithin,” “phospholipin,” “phospholipid,” and “phosphoaminolipid” have all been used or suggested as class names for fatty substances containing both phosphorus and nitrogen. Thudichum, who was a pioneer in the study of these substances, designated them by the term “phosphatide,” which is still the most widely used name. We shall employ it in this chapter.

Many writers have used “lecithin” as a general term for fatty substances containing phosphorus. Since “lecithin” denotes a single type of phosphatide, its use in a general sense should be discontinued.

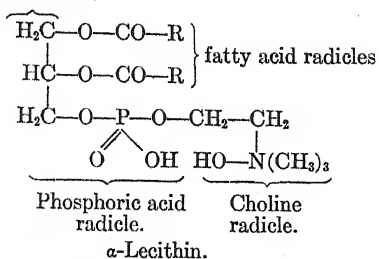
The term “phospholipin” is used widely in England, but it has been adopted by few investigators in other countries.

The Commission on the Reform of Biological Chemical Nomenclature² proposed that the term “phosphoaminolipide” be applied to fatty substances containing both phosphorus and nitrogen, *i. e.*, the phosphatides. The Commission suggested further that nitrogen-free derivatives of the phosphatides be called “phospholipides”; but in this country “phospholipid” is used widely as a general term, synonymous with “phosphatide.” In view of this confused situation it seems best to retain the old term “phosphatide,” concerning the meaning of which there is no question.

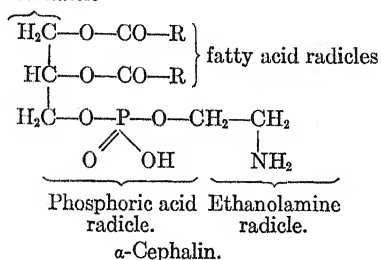
Classification and Structure.—Only three generic types of phosphatides have been identified with certainty. They are known as lecithin, cephalin, and sphingomyelin. The accompanying formulae (p. 123) are accepted by most investigators as representing the most probable structure of the **lecithins** and **cephalins**. They are based primarily on the behavior of these substances toward hydrolysis. Glycerophosphoric acid, fatty acid, and choline are formed when lecithins are hydrolyzed. Under the same treatment cephalins also yield glycerophosphoric acid and fatty acids but, instead of choline, ethanolamine (amino-ethyl alcohol) is produced. The discovery of this fact by MacLean¹⁶ provided a criterion for the absence of cephalin from lecithin preparations and *vice versa*. A pure lecithin gives no test for amino nitrogen by the Van Slyke method; the hydrolytic products of a pure cephalin contain no base which is precipitable with $PbCl_2$.

Since glycerophosphoric acid is obtained on controlled hydrolysis of lecithins and cephalins, it may be considered to be the nucleus of both types of substances. The fatty acid radicles can only be attached to the two free glycerol hydroxyls as esters and the base must then be linked to the phosphoric acid. Lecithins and cephalins may be regarded as neutral fats in which one fatty acid radicle has been replaced by a phosphoric acid-base group.

Glycerol radicle

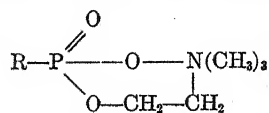


Glycerol radicle

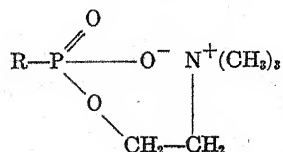


That the choline of lecithin and the ethanolamine of cephalin are combined in ester linkage through their alcoholic hydroxyl groups as shown, rather than as salts, was deduced from the fact that the bases are split off relatively slowly by acid. In the case of cephalins the ester linkage is established by the presence of a free amino group. Grün and Limpächer¹⁷ proved this structure by synthesizing distearyl lecithin and cephalin in which the ester linkage was known to be present. The synthetic lecithin agreed in properties with hydrolecithin which was obtained by reduction of egg lecithin. Moreover, the same authors¹⁸ showed that the choline salt of distearyl glycerophosphoric acid differed from synthetic lecithin in reacting as a monobasic acid, whereas synthetic and naturally occurring lecithins contain no titratable acid group. The analogous ethanolamine salt reacted as a dibasic acid, while cephalins have a single titratable acid group.

It might be expected, from the fact that choline is a strong base, that lecithins would exist as internal salts having the structure



or, according to modern views, the zwitterion form



The fact that lecithins contain no titratable acid group, when dissolved in organic solvents, supports the conception of an internal salt structure. The elementary analysis of the synthetic lecithin of Grün and Limpächer agreed with the internal salt formulation, but analyses of lecithins from natural sources have agreed better with the hydrated structure. It is doubtful, however, whether the presence or absence of one molecule of water can be detected in an analysis of a mixture of lecithins in which several different fatty acids are present. In aqueous suspension lecithins behave as amphoteric substances. According to Fabisch,¹⁹ lecithin sols have very weak buffering power against acids and alkalis, indicating that both acid and basic groups are strongly ionized.

Since ethanolamine is a relatively weak base the absence of an internal salt structure in cephalins might be expected. That this is the case is indicated by the presence of a titratable acid group and a free amino group in these substances.

The isolation of pure lecithin and cephalin is exceedingly difficult. The majority of preparations which have been studied were probably mixtures of the two substances. We are indebted especially to MacLean in England and P. A. Levene and his coworkers in this country for devising and refining methods by which fairly pure preparations have been obtained.

Elementary analyses of lecithin preparations have agreed fairly well with the theoretical values demanded by the formula; but until recently all preparations of cephalin, which have been analyzed, have yielded low values for carbon, hydrogen, nitrogen, and phosphorus. This result has been explained by assuming an absorption of oxygen by unsaturated groups in fatty acid radicles, or a loss of some fatty acid and base by hydrolysis during the process of isolation. The first explanation is improbable, since it has not been possible to identify oxy fatty acids in cephalin; and the second is also open to question, since the phosphorus percentage is low. If the average molecular weight were reduced by the loss of fatty acid and base, the percentage of phosphorus should be increased. Recently, Rudy and Page²⁰ have succeeded in isolating from brain small amounts of cephalin which gave elementary analyses agreeing satisfactorily with the theory, except for the phosphorus values, which were somewhat too low. Levene and West²¹ prepared saturated cephalin by hydrogenation. The elementary analysis of this substance agreed satisfactorily with the theory demanded by the formula given above. The difficulty in preparing cephalin itself which should comply with the theoretical composition remains unexplained.

Several different fatty acids have been isolated from lecithin and cephalin preparations. Levene and coworkers²² investigated a number of lecithins and cephalins from animal and plant sources. They found two saturated fatty acids (palmitic and stearic) and several unsaturated fatty acids in all of their preparations. Oleic, linolic,

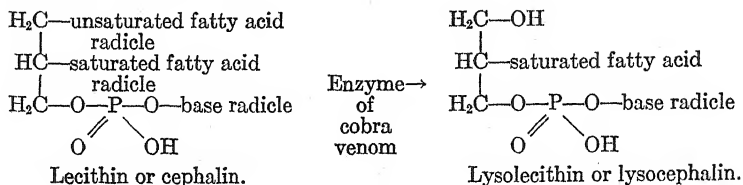
linolenic, and arachidonic acids were identified in various lecithins and cephalins of animal origin. Arachidonic acid was not found in plant lecithin. Levene and Rolf found that linolenic acid predominated in liver lecithin, but Snider and Bloor²³ obtained no linolenic acid at all from liver lecithin. Klenk and his collaborators²⁴ have reported the presence of highly unsaturated 22-carbon fatty acids in phosphatides from brain and liver. Page and Rudy²⁵ found fatty acids of the C_{22} series with four or more double bonds in brain cephalin, in confirmation of Klenk. It is interesting that unsaturated fatty acids with more than eighteen carbon atoms have been found in animal, but not in plant phosphatides.

The fact that several different fatty acids are present must mean that "pure" lecithin and cephalin, as they have been isolated, are not chemical individuals but mixtures of compounds which have the same general structure but differ in the fatty acid radicles which they contain. Ordinarily, in speaking of "lecithin" or "cephalin," a mixture of lecithins or cephalins is implied.

It is probable that each molecule of lecithin and cephalin contains one saturated and one unsaturated fatty acid radicle. Levene and Rolf²⁶ found that saturated and unsaturated fatty acids are present in approximately equimolecular proportions in lecithins. The same authors²⁷ brominated lecithins from liver and egg yolk and separated octa-, hexa-, tetra-, and di-bromolecithins. The elementary analyses of these bromine derivatives agreed fairly well with the theoretical composition of lecithins containing one molecule of saturated fatty acid (stearic or palmitic) and one molecule of brominated (from unsaturated) fatty acid. Stearic and palmitic acids were isolated from the bromolecithins.

Further evidence for the assumption that lecithins and cephalins contain one saturated and one unsaturated fatty acid radicle is found in the fact that the fully saturated distearyl lecithin and cephalin, which were synthesized by Grün and Limpächer, differ markedly from natural lecithins and cephalins in several of their properties. For instance, the synthetic phosphatides are much more resistant to oxidation and, in general, less soluble than natural lecithins and cephalins. These facts indicate that fully saturated lecithins and cephalins do not occur widely in nature. (Recently Merz²⁸ reported the isolation from brain of a small amount of a lecithin which was difficultly soluble in ether. Only 21 per cent of the fatty acids were unsaturated (oleic acid). The results indicate that fully saturated lecithins were present.) The formation of lysolecithin and lysocephalin by the action of cobra venom on lecithin and cephalin respectively supports this view. Cobra venom does not act on saturated lecithin and cephalin. Lysolecithin and lysocephalin contain only one fatty acid radicle, which is saturated. One glycerol hydroxyl is free. It follows from these facts that the enzymes of cobra venom split off one molecule of unsaturated fatty

acid from lecithin and cephalin molecules which contain one saturated and one unsaturated fatty acid radicle.



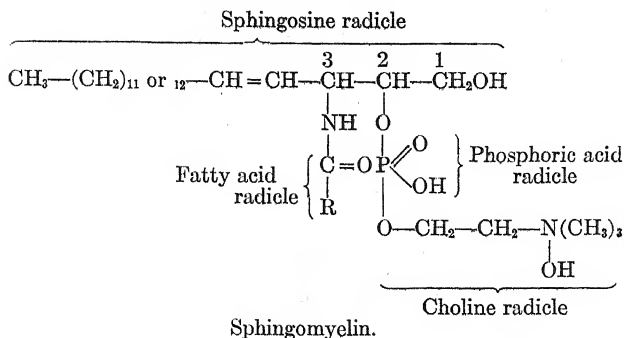
Since, as far as is known, cobra venom acts on all natural lecithins and cephalins, it seems highly probable that this is the normal structure.

Two types of isomerism are possible in lecithins and cephalins. One type depends on the position of the phosphoric acid-choline (or ethan-olamine) group in the molecule. In the formulae given these groups are attached to one of the α -carbon atoms of the glycerol radicle; but they may also be in the β -position. Until recently it was thought that only the α -form existed in nature. This view was based mainly on work of Willstätter and Lüdecke²⁹ who found that the barium salt of glycerophosphoric acid from lecithin possessed weak optical activity. This is possible only for the α -form which has an asymmetrical carbon atom. Karrer and Salomon³⁰ found that the barium salt of β -glycerophosphoric acid formed a difficultly soluble double salt with $\text{Ba}(\text{NO}_3)_2$. The α -form is not precipitated. With this method the authors showed that about 80 per cent of glycerophosphoric acid from egg and brain lecithins was the β -form. A crude cephalin preparation contained 45 per cent of β -glycerophosphoric acid. Karrer and Benz³¹ showed that no rearrangement of the α - to the β -form of glycerophosphoric acid took place during the process of isolation. Karrer and Salomon showed further that salts of α -glycerophosphoric acid from lecithin possess little if any optical activity although the dimethyl ester of α -glycerophosphoric acid dimethyl ether is optically active. They conclude that the rotation which Willstätter and Lüdecke observed must have been due to impurities. These results indicate that both the β - and α -forms of lecithin occur in nature with the former in larger amount.

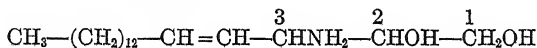
Lecithins and cephalins may also exhibit optical activity. This is obvious in the case of the α -forms. β -Lecithins and cephalins containing one saturated and one unsaturated fatty acid radicle also have the possibility of optical isomerism since the β -carbon carries four different groups and is asymmetric. All samples of lecithin, which have been examined, have shown dextrorotation.

Sphingomyelins differ markedly from lecithins and cephalins. On hydrolysis they yield fatty acids, choline, phosphoric acid, and a complex base: Sphingosine. No glycerol is present.

Sphingosine contains one unsaturated group, one amino group and two hydroxyl groups. Its structure has recently been investigated by Klenk.³² He acetylated (to protect the amino and hydroxyl groups)



and then oxidized the compound at the double bond with ozone. Myristic acid was one of the oxidation products. The other product (containing the acetylated amino and hydroxyl groups) was hydrolyzed to remove the acetyl groups and reduced to remove hydroxyl groups. α -Amino butyric acid was obtained. These reactions indicate that sphingosine has the structure



with eighteen carbon atoms in the molecule. Before the appearance of Klenk's work it was accepted generally that sphingosine contained seventeen carbon atoms. Lapworth³³ and Levene and West³⁴ found that tridecyclic (C_{13}) acid was formed on oxidation of the substance at the double bond. Many analyses of sphingosine and its derivatives by Levene and West³⁵ and Levene³⁶ agreed with a formula containing seventeen carbon atoms. Klenk points out that several of the analyses agree better with an 18-carbon structure. His own analyses of sphingosine derivatives are in accord with the formula he proposes. The final establishment of the structure of sphingosine must await future work.

The fatty and phosphoric acid groups must both be attached to sphingosine in the sphingomyelin molecule as no other structure is possible. Levene³⁶ isolated a compound of fatty acid and sphingosine (lignoceryl sphingosine) from the hydrolytic products of sphingomyelin. This compound contained no free amino group. The fatty acid must have been attached to the amino group of sphingosine by amide linkage and by inference this is the structure in sphingomyelin. Recently Thannhauser and Fränkel³⁷ have isolated lignoceryl sphingosine from liver. The phosphoric acid must be attached to one of the hydroxyls of sphingosine. The position shown in the formula is arbitrarily selected.

Lignoceric, stearic, and nervonic acids have been isolated from sphingomyelin preparations.³⁸ Evidently there are several (at least three) sphingomyelins just as there are several lecithins and cephalins.

Four stereo-isomers of sphingomyelin are possible since carbons 2 and 3 are asymmetric. The few samples, which have been studied, rotated polarized light to the right.

Physical Properties.—Freshly prepared lecithins and cephalins are white, waxy substances, but they are highly susceptible to oxidation in the air and, unless carefully protected, rapidly assume a yellow or brown color. They are very hygroscopic. Sphingomyelin, on the other hand, is a white, crystalline, and relatively stable substance.

The phosphatides are soluble in most of the "fat-solvents" but differ from neutral fats in several important particulars. When pure they are all insoluble in acetone; cephalin and sphingomyelin are insoluble in cold alcohol; sphingomyelin is insoluble in ether. These properties are utilized in the separation of phosphatides from other fatty substances and from each other. Unfortunately, complete separation on the basis of these solubility relationships is difficult since fatty substances exert a solvent action on each other. For instance, in the separation of phosphatides from neutral fats by means of acetone, appreciable amounts of phosphatide may be carried into the acetone by the solvent action of the dissolved fat.

The phosphatides form colloidal solutions in water. This property is thought to be of great importance in the metabolism of the fatty substances since it provides a method for their transport in aqueous body fluids. The phosphatides are precipitated from aqueous colloidal solution by acids and most neutral salts.

Price and Lewis³⁹ showed, by studies of the electrophoretic mobility of lecithin sols, that the point of reversal of charge, *i. e.*, the effective iso-electric point, is at pH 2.7. The authors calculated from the dissociation constants of choline and substituted glycerophosphoric acid that the theoretical iso-electric point of lecithin (where the positive and negative charges on the zwitterions are equal) is at pH 5.2. They suggested that the discrepancy is caused by a complex charge, part of which is due to ionization and part to adsorption of hydroxyl ions by the fatty portions of the dispersed particles of the sol.

Chemical Properties and Reactions.—The phosphatides are hydrolyzed readily by acids and alkalis into their constituents. Enzymes having the property of splitting phosphatides are distributed widely in animal and plant fluids and tissues. Cobra venom contains enzymes with the property of splitting off one molecule of fatty acid from lecithin and cephalin. The resultant compounds—lysolecithin and lysocephalin—have the property of hemolyzing red blood cells. As noted above, the single fatty acid radicle of these compounds is always saturated.

The phosphatides form addition compounds with many inorganic salts. The best known are the cadmium chloride compounds which have been utilized extensively in the separation and purification of the phosphatides. All three cadmium chloride compounds are insoluble in cold alcohol, but they differ in their solubility in ether; lecithin and sphingomyelin cadmium chlorides are insoluble while cephalin cadmium chloride is soluble. The addition of magnesium chloride assists in the precipitation of lecithin and cephalin from ether by acet-

one, probably through the formation of insoluble compounds. It is widely used in the separation of phosphatides from other fatty substances.

In benzene-alcoholic solution, cephalin behaves as a monobasic acid and may be titrated with alkali. Rudy and Page²⁰ have utilized this fact in distinguishing cephalin from lecithin, which does not react as an acid. These authors have also prepared a true barium salt of cephalin (as distinguished from the addition compounds discussed above) by treating a cephalin solution with barium hydroxide.

Lecithin and cephalin enter into the reactions which are typical for unsaturated fatty acids; they add bromine, iodine, or hydrogen, and are readily oxidized. Hydrogen unites (in the presence of catalysts such as colloidal palladium) at the double bonds of the unsaturated fatty radicles with the formation of hydrolecithin and hydrocephalin. These saturated phosphatides are crystalline, nonhygroscopic, stable substances. Hydrolecithin is insoluble in water and acetone, slightly soluble in ether, and sparingly soluble in alcohol. Synthetic distearyl lecithin has similar properties.

Lecithin and cephalin are oxidized spontaneously and rapidly when exposed to air and light. Products are formed which are insoluble in alcohol and ether but soluble in water. It is this tendency to oxidation, more than any other factor, which makes the preparation of pure lecithin and cephalin so difficult. Oxidation occurs at the double bonds of the unsaturated fatty acid radicles. This is shown by the fact that hydrolecithin and hydrocephalin are stable toward air and light. The degree of oxidation of lecithin and cephalin may be followed by the iodine number which decreases as the double bonds are oxidized.

FATTY SUBSTANCES CONTAINING GALACTOSE (CEREBROSIDES)

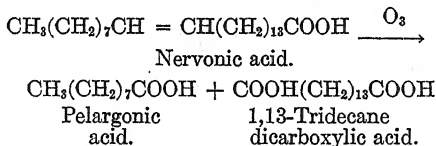
Nomenclature.—As in the case of the phosphatides, many different names have been suggested for the class of substances which is to be considered in this section. They were called "cerebrosides" by Thudichum who published the first extensive studies of these substances in 1874.⁴⁰ Other names which have been proposed are "cerebrogalactoside," "galactoside," "galactolipin," and "galactolipid." None of these terms has supplanted "cerebroside" from general use.

Classification and Structure.—Three cerebrosides have been isolated. These are *kerasin*, *phrenosin* (or *cerebron*), and *nervon*. They are thought to be identical in general structure, and to differ only in the fatty acid radicles which they contain. On complete hydrolysis each yields sphingosine, galactose, and a fatty acid. They contain no glycerol or phosphorus.

A substance, called *psychosin*, has been obtained from each of the cerebrosides by splitting off fatty acid by long hydrolysis with barium hydroxide. Psychosin yields sphingosine and galactose on complete hydrolysis. It contains a free amino group and does not reduce

tetracosanic acid, and the other of which melted about 11 degrees lower, although it also contained twenty-four carbon atoms. The lower melting fraction did not react to oxidizing agents like a branched-chain acid, and Brigl and Fuchs suggested that it had a normal structure but differed from the higher melting acid by an unknown type of isomerism. Levene, Taylor, and Haller,⁴⁵ using the technic of Brigl and Fuchs (fractional crystallization from pyridine) were unable to separate lignoceric acid from peanut oil and kersin into components. All fractions melted at 80°–81° C. They retained the opinion of Levene and West that lignoceric acid is a single substance isomeric with normal tetracosanic acid. If Levene's opinion is correct, lignoceric acid is the only branched-chain fatty acid which has been found to occur naturally in plants and animals.

Klenk believes that lignoceric acid has a normal structure. In 1927 he confirmed partially⁴⁶ the results of Brigl and Fuchs. He fractionated a sample of lignoceric acid (from kersin—melting point, 80.5°–81.5° C.) by precipitation with lithium acetate from hot alcohol. He was able to separate several fractions, with melting points varying between 82° and 84° C. Their combined weight was about one half that of the original sample. A small fraction melting at 54°–56° C. and another melting at 77°–78° C. were obtained. One small fraction (10–15 per cent of the original sample) with a melting point of 83°–84° C. was compared with reduced nervonic acid. This acid (from the cerebroside nervon) contains one double bond. On oxidation with ozone it gave pelargonic acid and a dibasic acid, which Klenk⁴⁷ believed to be identical with synthetic 1,13-tridecane dicarboxylic acid.

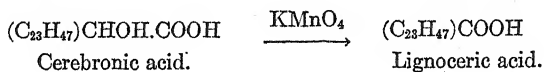


This reaction indicates that nervonic acid has a normal structure. On reduction nervonic acid gave a saturated acid, melting at 85°–85.5° C., which was shown to be identical with normal tetracosanic acid.⁴⁸ Mixtures of this reduced nervonic acid and the fraction from lignoceric acid, melting at 83°–84° C., showed no depression in melting point. The methyl esters of the two substances melted at the same point (59.5° C.) and no depression in melting point was caused by mixing one with the other.

Klenk believes that the foregoing evidence shows beyond doubt that lignoceric acid has a normal structure; but his results indicate also that it is a mixture, of which only part is identical with reduced nervonic acid, and, therefore, with normal tetracosanic acid. The nature of the lower melting acid or acids which comprise the remainder of the mixture remains unexplained. If lignoceric acid is a mixture, it is remarkable that all samples, which have been isolated from several

plant and animal sources, give the same melting point and are apparently identical. Further work is necessary to establish the nature of lignoceric acid beyond any doubt.

The Fatty Acid of Phrenosin is Cerebronic Acid.—The nature of this substance is also the subject of a controversy between Levene and Klenk. In 1904 Thierfelder⁴⁹ presented evidence indicating that it contained twenty-five carbon atoms and one hydroxyl group. In 1912 Levene and Jacobs⁵⁰ obtained lignoceric acid on oxidation with KMnO_4 and concluded that the hydroxyl group was in the α -position.

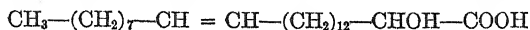


Further proof for this formulation was obtained by Levene and Taylor⁵¹ who converted lignoceric acid into cerebronic acid. In 1928 Klenk⁵² obtained normal C_{23} (tricosanic) acid, instead of the C_{24} acid which Levene and Jacobs had found on oxidation of cerebronic acid, and concluded that the latter was an α -hydroxy C_{24} acid. In further support of this view, Klenk reported⁵³ that cerebronic acid gave lignoceric acid on reduction.

Soon after the appearance of Klenk's papers, Taylor and Levene⁵⁴ presented data which were neither in agreement with their own earlier work nor with Klenk's results. They found that the oxidation product of cerebronic acid was a *mixture* of acids which could be separated into several fractions with different properties by fractional distillation of methyl esters. A fraction, which corresponded to Klenk's tricosanic acid, was obtained, but it was shown to be a mixture. In 1933 Klenk and Diebold⁵⁵ reaffirmed Klenk's earlier conclusion that tricosanic acid is the principal oxidation product of cerebronic acid. However, about one third of the total amount had a molecular weight definitely below that of tricosanic acid, in agreement with the results of Taylor and Levene. Recently Levene and his coworkers⁵⁶ have extended their studies of the oxidation products of cerebronic acid. The results have confirmed them in the opinion that cerebronic acid is a mixture of hydroxy acids.

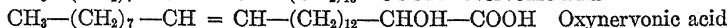
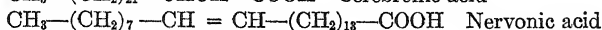
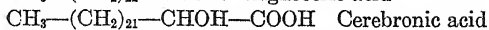
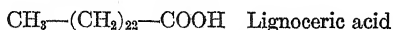
Nervon was first isolated by Klenk in 1925.⁵⁷ It does not appear to have been studied by other investigators. Its fatty acid (**nervonic acid**) was found to be a mono-unsaturated normal tetracosanic acid. The double bond is between carbons 15 and 16.

The existence of another cerebroside, **oxynervon**, has been deduced by Klenk from his discovery of an acid, oxynervonic acid, in the hydrolytic products of a cerebroside mixture.⁵² The cerebroside itself has not been isolated. Oxynervonic acid has one double bond and one hydroxyl group. On reduction it gives cerebronic acid. Pelargonic acid, $\text{CH}_3(\text{CH}_2)_7\text{COOH}$, is formed on oxidation with ozone. On the basis of these properties Klenk assigns the following formula to oxynervonic acid:



This formula cannot be regarded as established with certainty since it is based largely on its close relationship to cerebronic acid, of which the structure is still in doubt.

In summary, Klenk believes that the four fatty acids, which have been found in cerebrosides, are closely related C_{24} acids with a straight-chain structure.



It is unfortunate that the evidence of Levene and his coworkers makes it necessary to await further work before accepting this relatively simple conception.

Physical Properties.—The cerebrosides are insoluble in ether, petroleum ether, and water. They dissolve readily in cold pyridine. They are also soluble in hot alcohol, benzene, acetone and chloroform, but precipitate out on cooling in the form of liquid crystals, which solidify to a pseudo-crystalline, white, amorphous powder. Under the microscope the small, individual particles are seen to be globular masses showing a roset-like structure. It is possible to transform phrenosin into a true crystalline form⁵⁹ but kersin and nervon have not been crystallized.

The cerebrosides do not show true melting points. On heating they go first into a liquid, anisotropic, crystalline state, and then, at higher temperatures, they fuse to a clear liquid. On cooling from the fused state, anisotropic liquid crystals suddenly appear under crossed Nicols in the polarizing microscope. When the liquid crystals are viewed through a selenite plate, a characteristic difference in the color pattern of phrenosin and kersin is seen.⁵⁸ This property has been used generally as a test for the purity of these substances, but nervon gives the same picture as kersin.

Chemical Properties and Reactions.—The cerebrosides are hydrolyzed by boiling acid (dilute sulfuric acid is usually employed) into galactose, sphingosine, and fatty acid. They are quite resistant to splitting by alkali. In fact, in one method of preparation the cerebrosides are freed from phosphatides by mild hydrolysis with barium hydroxide. The phosphatides are saponified but the cerebrosides are not appreciably attacked. No enzymes with definite ability to split cerebrosides have been found.

The cerebrosides take up halogens at the double bond of the sphingosine radicle. Nervon (and presumably oxynervon) also add halogen at the double bond of the fatty acid radicle.

The cerebrosides possess several asymmetric carbon atoms: In the galactose radicle; in the sphingosine radicle; and, in the case of phrenosin, in the fatty acid radicle (the carbon carrying the hydroxyl

group is asymmetric). Phrenosin is dextrorotatory; kersin and nervon are levorotatory.

The cerebrosides give carbohydrate tests due to the galactose radicle. They form derivatives, such as methyl, acetyl, and benzoyl compounds, which are typical of substances containing hydroxy groups. For instance, phrenosin, which has six hydroxyl groups, forms a hexaacetyl derivative. They are precipitated from alcoholic solution by lead acetate in the presence of ammonia. Klenk⁵⁷ utilized this property in the preparation of nervon.

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REFERENCES

General References

- Lewkowitsch, J.: *Chemical Technology and Analysis of Oils, Fats, and Waxes* (1921).
 Leathes, J. B., and Raper, H. S.: *The Fats* (1925).
 Bloor, W. R.: *Biochemistry of the Fats*, *Chem. Rev.*, **2**, 243 (1925).
 Grün, A., und Halden, W.: *Analyse der Fette und Wachse, sowie der Erzeugnisse der Fettindustrie. Band II. Systematik, Analysenergebnisse und Bibliographie der natürlichen Fette und Wachse* (1929).
 Levene, P. A., and Rolf, I. P.: *Structure and Significance of the Phosphatides*, *Physiol. Rev.*, **1**, 327 (1921).
 MacLean, H., and MacLean, I. S.: *Lecithin and Allied Substances. The Lipins* (1927).
 Thierfelder, H., und Klenk, E.: *Die Chemie der Cerebroside und Phosphatide* (1930).

Specific References

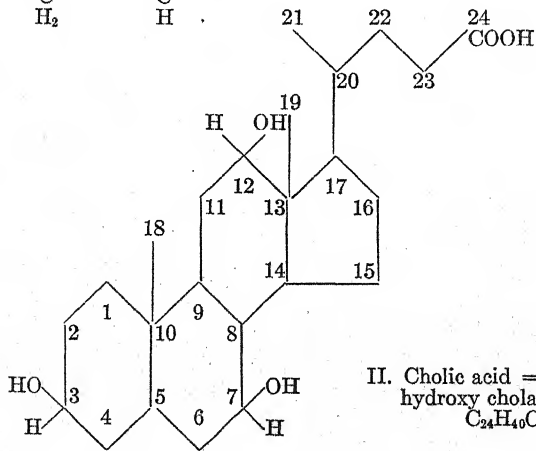
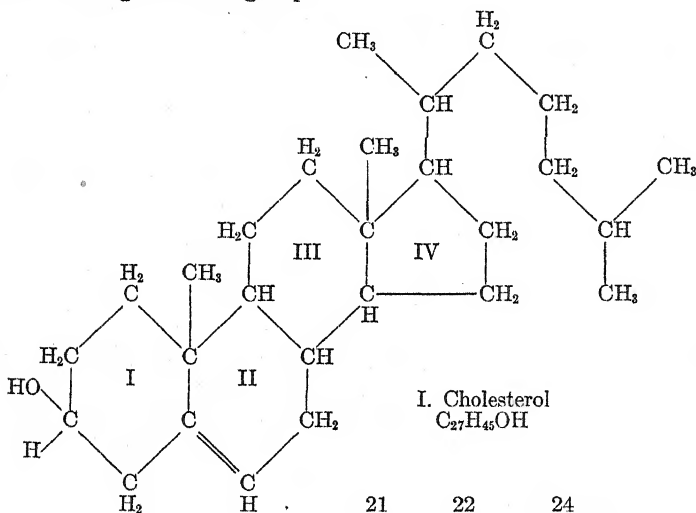
1. Gérard, J.: *Ind. Eng. Chem.*, **15**, 1082 (1923).
2. Seidell, A.: *Ind. Eng. Chem.*, News Ed., **6**, No. 17, 3 (1928).
3. Rosenbloom, J., and Gies, W. J.: *Biochem. Bull.*, **1**, 51 (1911-12).
4. MacLean, H.: *Lecithin and Allied Substances. The Lipins* (1918), p. 2.
5. Anderson, R. J.: *J. Biol. Chem.*, **97**, 639 (1932).
6. Bosworth, A. W., and Brown, J. B.: *J. Biol. Chem.*, **103**, 115 (1933).
7. Haworth, R. D.: *J. Chem. Soc.*, 1456 (1929).
8. Hilditch, T. P.: *J. Chem. Soc.*, 1828 (1926).
9. Averill, H. P., Roche, J. N., and King, C. G.: *J. Am. Chem. Soc.*, **51**, 866 (1929).
10. Mattill, H. A.: *J. Biol. Chem.*, **90**, 141 (1931).
11. Robinson, H. E., Roche, J. N., and King, C. G.: *J. Am. Chem. Soc.*, **54**, 705 (1932).
12. Abderhalden, E., und Eichwald, E.: *Ber.*, **48**, 1847 (1915).
13. Hilditch, T. P., and Lea, C. H.: *J. Chem. Soc.*, 3106 (1927).
14. Hilditch, T. P., and Jones, E. C.: *J. Chem. Soc.*, 805 (1932).
15. Anderson, R. J., and Newman, M. S.: *J. Biol. Chem.*, **101**, 499 (1933).
16. McLean, H.: *Biochem. J.*, **9**, 351 (1915).
17. Grün, A., und Limpächer, R.: *Ber.*, **59**, 1350 (1926); **60**, 151 (1927).
18. Grün, A., und Limpächer, R.: *Ber.*, **59**, 1345 (1926).
19. Fabisch, W.: *Biochem. Z.*, **242**, 121 (1931).
20. Rudy, H., und Page, I. H.: *Z. physiol. Chem.*, **193**, 251 (1930).
21. Levene, P. A., and West, C. J.: *J. Biol. Chem.*, **35**, 285 (1918).
22. Levene, P. A., and Ingvaldsen, T.: *J. Biol. Chem.*, **43**, 359 (1920); Levene, P. A., and Simms, H. S.: *Ibid.*, **48**, 185 (1921); **51**, 285 (1922); Levene, P. A., and Rolf, I. P.: *Ibid.*, **46**, 193, 353 (1921); **51**, 507 (1922); **54**, 91 (1922); **62**, 759 (1924-25); **65**, 545 (1925); **67**, 659 (1926); **68**, 285 (1926).
23. Snider, R. H., and Bloor, W. R.: *J. Biol. Chem.*, **99**, 555 (1932-33).
24. Klenk, E.: *Z. physiol. Chem.*, **192**, 217 (1930); **200**, 51 (1931); Klenk, E., und v. Schoenebeck, O.: *Ibid.*, **209**, 112 (1932).
25. Page, I. H., und Rudy, H.: *Z. physiol. Chem.*, **205**, 115 (1932).
26. Levene, P. A., and Rolf, I. P.: *J. Biol. Chem.*, **46**, 193 (1921).
27. Levene, P. A., and Rolf, I. P.: *J. Biol. Chem.*, **67**, 659 (1926).

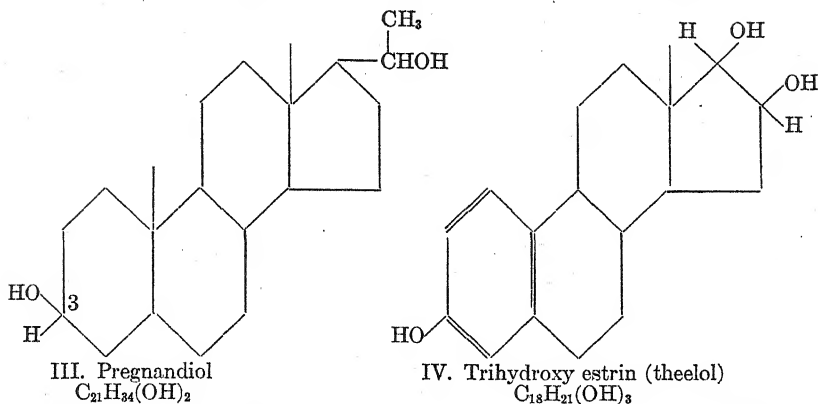
28. Merz, W.: *Z. physiol. Chem.*, **196**, 10 (1931).
29. Willstätter, R., und Lüdecke, K.: *Ber.*, **37**, 3753 (1904).
30. Karrer, P., and Salomon, H.: *Helv. chim. Acta*, **9**, 3 (1926).
31. Karrer, P., and Benz, P.: *Helv. chim. Acta*, **10**, 87 (1927).
32. Klenk, E.: *Z. physiol. Chem.*, **185**, 169 (1929); Klenk, E., and Diebold, W.: *Ibid.*, **198**, 25 (1931).
33. Lapworth, A.: *J. Chem. Soc.*, **103**, 1029 (1913).
34. Levene, P. A., and West, C. J.: *J. Biol. Chem.*, **16**, 549 (1913-14); **18**, 481 (1914).
35. Levene, P. A., and West, C. J.: *J. Biol. Chem.*, **24**, 63 (1916).
36. Levene, P. A.: *J. Biol. Chem.*, **24**, 69 (1916).
37. Thannhauser, S. J., und Fränkel, E.: *Z. physiol. Chem.*, **203**, 183 (1931).
38. Merz, W.: *Z. physiol. Chem.*, **193**, 59 (1930).
39. Price, C. W., and Lewis, W. C. M.: *Trans. Faraday Soc.*, **29**, 775 (1933).
40. Thudichum, J. L. W.: Rep. Med. Officer Privy Council. Loc. Gov. Board, New Series, **3**, 113 (1874).
41. Klenk, E.: *Z. physiol. Chem.*, **153**, 74 (1926).
42. Meyer, H., Brod, L., und Soyka, W.: *Monatsh.*, **34**, 1113 (1913).
43. Levene, P. A., and West, C. J.: *J. Biol. Chem.*, **18**, 477 (1914).
44. Brigl, P., und Fuchs, E.: *Z. physiol. Chem.*, **119**, 280 (1922).
45. Levene, P. A., Taylor, F. A., and Haller, H. L.: *J. Biol. Chem.*, **61**, 157 (1924).
46. Klenk, E.: *Z. physiol. Chem.*, **166**, 268 (1927).
47. Klenk, E.: *Z. physiol. Chem.*, **166**, 287 (1927).
48. Klenk, E.: *Z. physiol. Chem.*, **157**, 283 (1926).
49. Thierfelder, H.: *Z. physiol. Chem.*, **43**, 21 (1904-05).
50. Levene, P. A., and Jacobs, W. A.: *J. Biol. Chem.*, **12**, 381 (1912).
51. Levene, P. A., and Taylor, F. A.: *J. Biol. Chem.*, **52**, 227 (1922).
52. Klenk, E.: *Z. physiol. Chem.*, **174**, 214 (1928).
53. Klenk, E.: *Z. physiol. Chem.*, **179**, 312 (1928).
54. Taylor, F. A., and Levene, P. A.: *J. Biol. Chem.*, **84**, 23 (1929).
55. Klenk, E., und Diebold, W.: *Z. physiol. Chem.*, **215**, 79 (1933).
56. Levene, P. A., and Heymann, K.: *J. Biol. Chem.*, **102**, 1 (1933); Taylor, F. A., and Levene, P. A.: *Ibid.*, **102**, 535 (1933); Levene, P. A., and Yang, P. S.: *Ibid.*, **102**, 541 (1933).
57. Klenk, E.: *Z. physiol. Chem.*, **145**, 244 (1925).
58. Rosenheim, O.: *Biochem. J.*, **8**, 110 (1914).
59. Loening, H., und Thierfelder, H.: *Z. physiol. Chem.*, **68**, 464 (1910); Levene, P. A., and Heymann, K.: *J. Biol. Chem.*, **102**, 1 (1933).

CHAPTER IV

STEROLS, BILE ACIDS AND RELATED COMPOUNDS

THE groups of compounds discussed in this chapter would not seem to have much in common for one who views their occurrence and function in various organisms and organs. But the investigation of their chemical structure has led to results which demand their classification under one heading. A condensed isocyclic system, consisting of three six-membered and one five-membered ring, forms the architectural principle common to them and suggests genetic relationships which remain to be proved by biological experiments. The formulas of the typical representatives, **cholesterol (I)**, **cholic acid (II)**, **pregnandiol (III)**, and **trihydroxy estrin (IV)**, illustrate the identity of the carbon skeleton throughout this group.

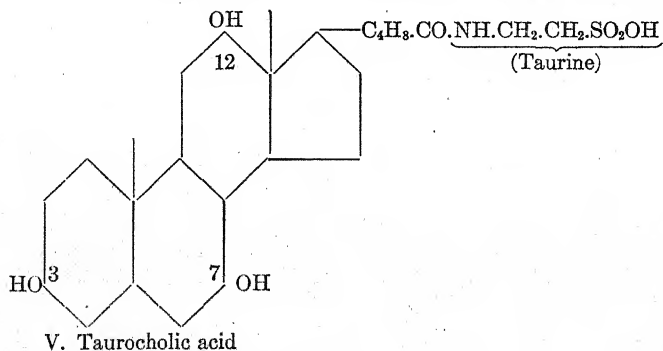




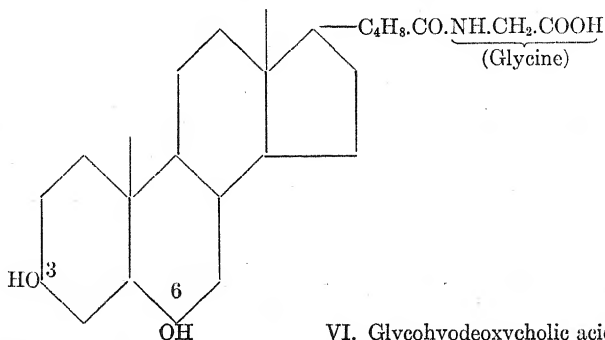
The rings are designated by Roman numerals, and the carbon atoms bear the numbers 1–17; the eighteenth and nineteenth carbon atoms are methyl groups, while the numbers above 20 are used for the side-chain.

While representatives of the sterols and bile acids were recognized as chemical individuals in the early nineteenth century, the successful investigation of their molecular structure is of relatively recent date.¹ The present structural formulas were established only quite recently by Rosenheim and King.² As the work of their predecessors was based on an incorrect formulation, the more than 500 derivatives described before 1932 have to be reformulated and partly renamed.³ The majority of these derivatives has not been found in nature and the study of their physiologic action has been neglected.

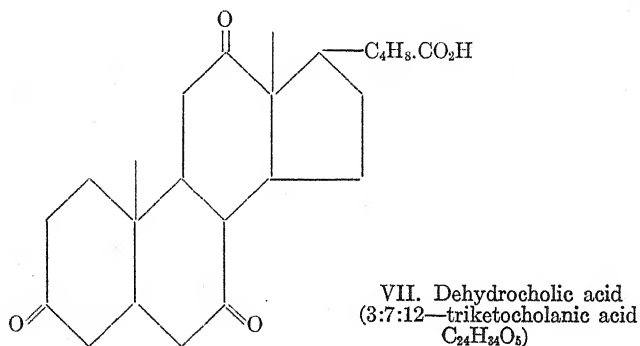
Methods of Investigation.—Oxidation, reduction, halogenation, dehydrogenation and dehydration were the most important methods used in these structural studies. **Cholic acid (II)** is a saturated trihydroxymonobasic acid. The carboxyl group is in position 24 at the end of the side-chain, the hydroxy groups are on the carbon atoms 3 of ring I, 7 of ring II, and 12 of ring III. It is the most important constituent of the bile of nearly all vertebrate animals where it is present in “conjugated” form, *i. e.*, in a peptide-like linkage with the amino acids glycine or taurine (V). Other bile acids are the dihydroxy acids:



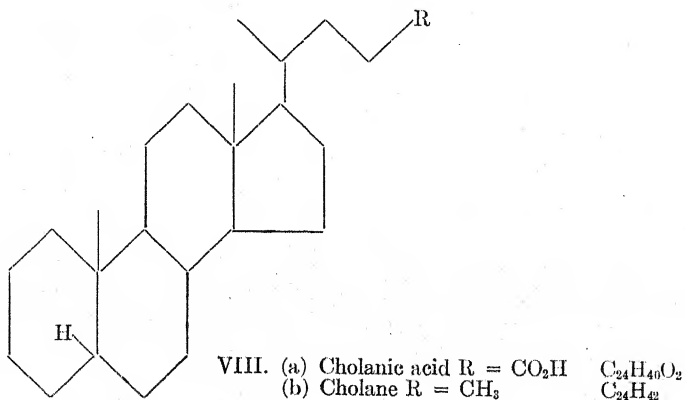
Deoxycholic acids with hydroxy groups in 3 and 12, *hyo*-deoxycholic from hog bile (3,6), *cheno*-deoxycholic acids (3,7) from goose and human bile, all answering the formula $C_{24}H_{40}O_4$, and all occurring in conjugation with glycine (VI) or taurine, and lithocholic acid, a



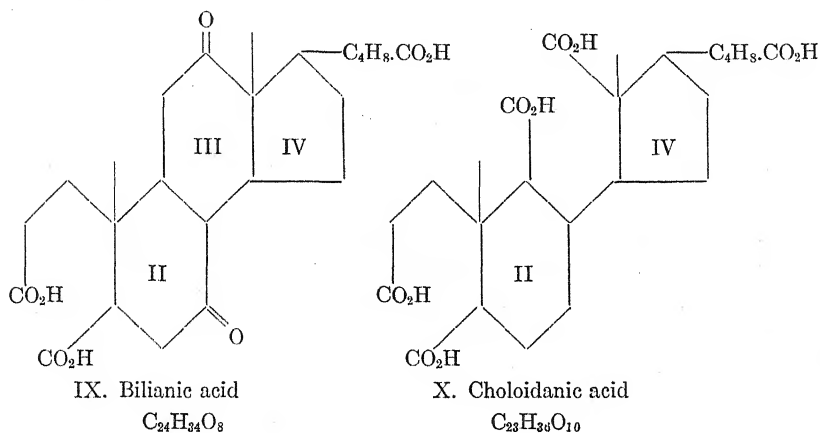
3: monohydroxy acid. Mild oxidation of the hydroxy groups yields keto-acids, the so-called "dehydro" acids as, *e. g.*, dehydrocholic acid



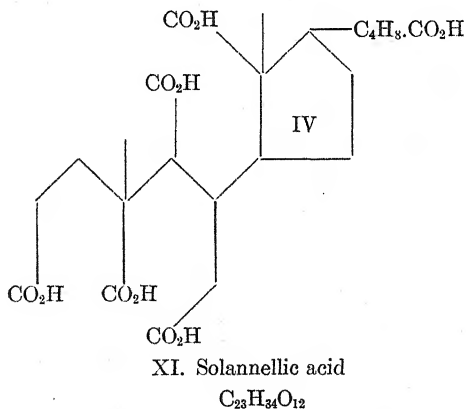
(VII). Reduction of the carbonyl groups in the dehydro-acids leads to the parent acid cholanic acid, VIII(a), a monovalent acid corre-



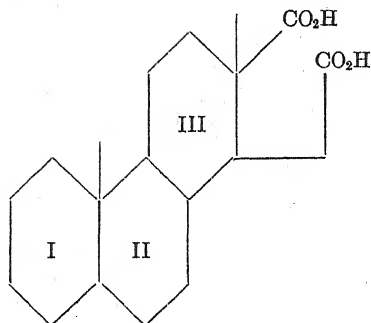
sponding to the hydrocarbon $C_{24}H_{42}$ cholane [VIII(b)]. Stronger oxidizing agents cause the rupture of the various rings near the carbonyl groups, usually yielding two carboxyl groups at the point of rupture and resulting in the formation of polyvalent acids, such as bilianic acid



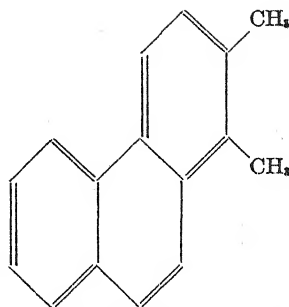
(IX) from dehydrocholic acid, choloidanic acid (X) from dehydrodeoxycholic acid (3:12-diketocholanic acid), with rupture of rings I and III, and solannellic acid (XI, solus annellus, one ring only)



with rupture of rings I, II and III (and loss of carbon atom 4). Gradual degradation of the side-chain by various methods yields, among other compounds, the tricyclic aetiobilianic acid (XII) which can be further converted into 3:4-dimethylphenanthrene (XIII).



XII. Aetiobilanic acid
 $C_{19}H_{30}O_4$

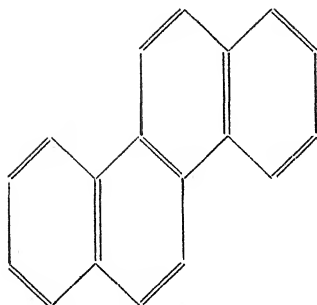


XIII. 3:4-Dimethylphenanthrene
 $C_{14}H_{14}$

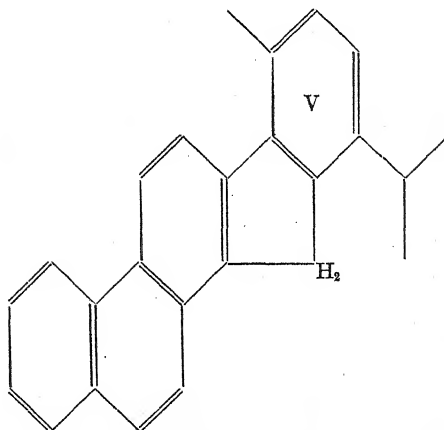
Cholesterol (I), the most important of animal sterols, is a mono-unsaturated tetracyclic secondary alcohol, with the hydroxyl group on carbon atom 3, and the double bond between carbon atoms 5 and 6; rings I and II, containing these, can be modified and eventually disrupted, yielding a great many derivatives. Degradation of the side-chain (elimination of three carbon atoms) leads into the series of the so-called "allocholanolic acid," a stereo-isomer of cholanolic acid, VIII (a). Fragments of the side-chain of sterols, obtained by oxidative destruction of the molecule, have been identified with well-known aliphatic substances such as acetone, methyl-isohexyl-ketone, methyl-isopropyl-acetaldehyde (from ergosterol) and ethyl-isopropyl-acetaldehyde (from stigmaterol).

Other products with the identical tetracyclic carbon skeleton, but with modified side-chains, are scymnol which takes the place of the bile acids in the bile of the lowest vertebrates (elasmobranchii: shark). In contrast to the bile acids of higher animals, scymnol is conjugated with sulfuric acid by esterification of an alcoholic hydroxyl group.⁴ Bufotalin, the principle of toad poison, is a derivative of bufocholanolic acid, another stereo-isomer of cholanolic acid; it is conjugated by an ester-like linkage with suberic acid, whose other carboxyl group in turn forms a peptide with a molecule of arginine.⁵

Further degradation of the side-chain of cholanolic acid leads to the formula (III) of **pregnandiol**, a substance of no known physiologic activity, isolated from the urine of pregnant women. It is related to the corpus luteum hormones, recently isolated in crystalline form. In the **female sex hormone**, one observes three double bonds in ring I, imparting an aromatic character to this ring and a phenolic character to the hydroxy group in position 3. **Equilenine**, another estrogenic compound, isolated in the urine of pregnant mares, contains five double bonds in ring I and II as in naphthalene.⁶ Experimental dehydrogenation of cholesterol or cholic acid by palladium charcoal or selenium leads (with certain side reactions) to substances with a partially or completely aromatic skeleton such as **chrysene** (XIV) or $C_{25}H_{22}$



XIV. Chrysene
 $C_{18}H_{12}$



XV. Hydrocarbon $C_{25}H_{22}$ according to Rosenheim and King

(XV), while the phenanthrene derivative (XIII) mentioned above is obtainable by similar methods from the sex hormone.⁷ These reactions point to a relationship to compounds like retene, abietic acid and others found in natural resins. A genetic relationship between sterols and terpenoid substances has been suspected since the work of Lachinov,⁸ but this connection has never been established experimentally. (See also Chapter XXX.)

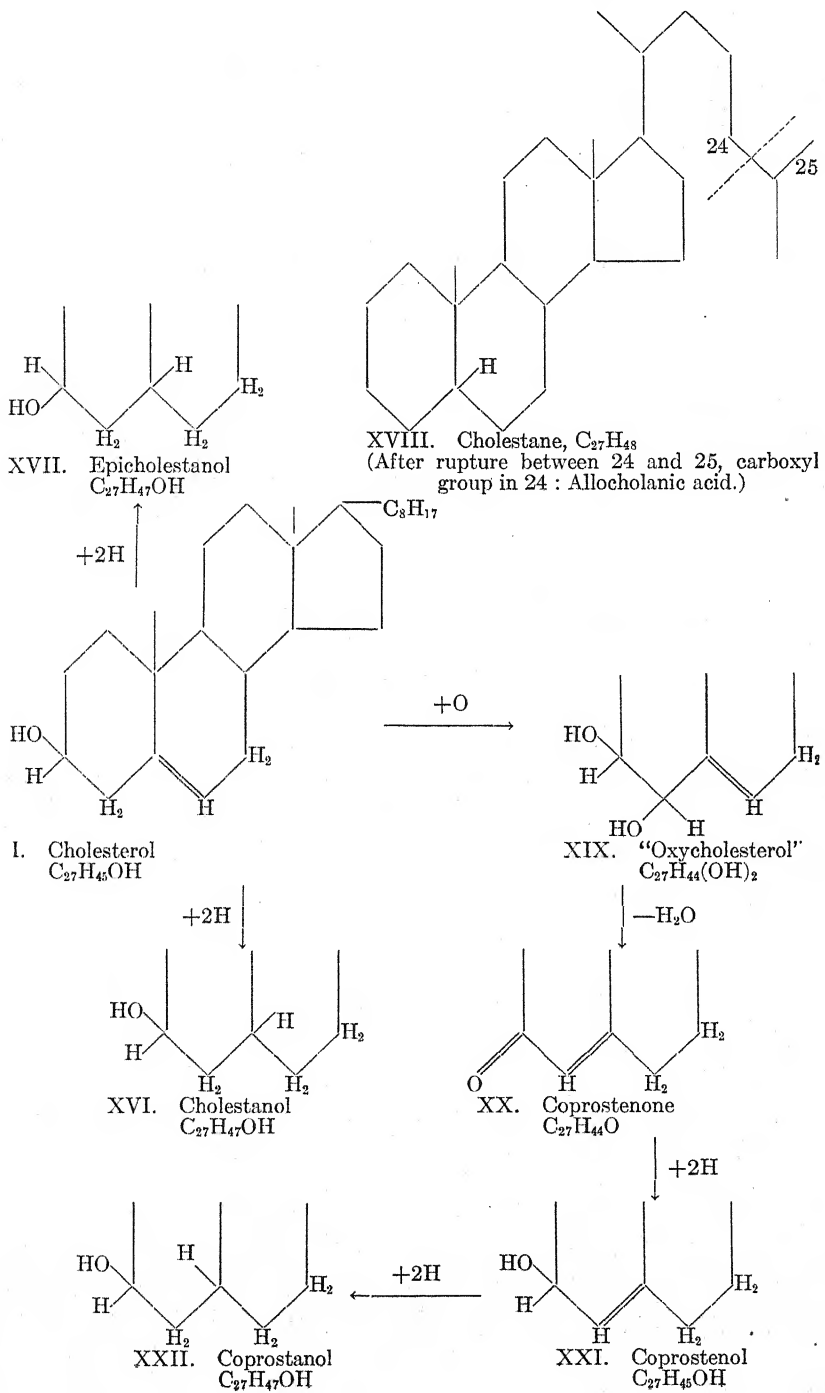
Another group of hydrocarbons, which bears a certain resemblance to the estrogenic hormones, are the **carcinogenic compounds** prepared by Cook, Dodds, *et al.*,⁹ which exert a noticeable estrogenic action. Such substances (*e. g.*, 1:2:5:6-dibenzanthracene) are also found in certain tar fractions, and are responsible for the experimental generation of tumors by tar. As the "cell proliferation which characterizes the estrous state is in some respects reminiscent of the early stages of malignant growth,"⁹ one may suspect that dehydrogenation of sterols is the physiologic method for the production of the closely related estrogenic hormones; but that further dehydrogenation by a faulty mechanism gives rise to carcinogenic substances.¹⁰ (Chapter XXX.)

Cholesterol was discovered in the unsaponifiable portion of animal and human fat, and as the main constituent of human gall stones; hence its name (due to Chevreul¹¹ from the Greek $\chiολη$ = bile and $\sigmaτερεος$ = solid). It constitutes a considerable part of the lipoids of the brain¹² and contributes to the lipid fraction of all organs. Human blood serum contains 150–200 mg. per 100 cc., but this figure may rise to ten times its value under pathologic conditions. Sixty to 100 mg. per 100 cc. are found in normal liver bile, multiple amounts in the concentrated gallbladder bile. As a rule, cholesterol is accompanied by its esters, except in the red blood corpuscles and in bile, which contain free cholesterol only.

Pure cholesterol crystallizes in oblique platelets of the monoclinic system, with one corner frequently missing. It melts at 149° C. and has a specific rotation of -41° (in chloroform). Some of its typical color reactions are the Liebermann-Burchard reaction (green color with acetic anhydride and concentrated sulfuric acid), Salkowski's reaction (purple color with chloroform and sulfuric acid), Schiff's reaction (xantho reaction with nitric acid and ammonia), Lifschütz's reaction (oxidation with benzoyl peroxide to "oxycholesterol," then typical color changes with sulfuric acid), and Bernouilli's reaction (amber coloration with acetyl chloride or benzoyl chloride).¹⁴ The Liebermann-Burchard reaction is widely used for the colorimetric estimation of cholesterol (*e. g.*, in Bloor's method¹⁵). Gravimetric determinations of cholesterol are based on the formation of alcohol-insoluble cholesterol-digitonide. The formation of addition compounds with digitonin is not confined to cholesterol, but is shared by several of its derivatives (though not the esters), by other natural sterols and by some members of the related saponin group.¹⁶ The formation of insoluble digitonides allows one to distinguish between sterols with the hydroxyl on C_3 in the same position as in cholesterol (*e. g.*, in dihydrocholesterol (XVI), beta-cholestanol) and those of the so-called "epi" series with the hydroxyl in the opposite direction as epicholestanol (epsilon-cholestanol, XVII).

That cholesterol on account of its $>\text{CHOH}$ group is a secondary alcohol was recognized by Berthelot who prepared cholesteryl acetate, propionate, etc.¹⁷ The cholesterol esters of the higher fatty acids are of special interest since they accompany cholesterol in nature. Cholesteryl palmitate and cholesteryl oleate have been isolated from blood serum.¹⁸ All cholesterol esters display spectacular iridescence when solidifying from the melted state. This phenomenon is caused by the existence of an anisotropic or "liquid crystalline" phase as a more or less transitory state of aggregation. In fact, this peculiar state of aggregation was discovered first in the cholesterol esters. These esters exert a peculiar influence on colloidal systems, and their own solubility is influenced by various colloids. Thus, their presence in tissues and fluids is an important factor in many problems of physiology and pathology.

The iodine number of cholesterol, like that of other cyclic compounds, cannot be determined accurately by the customary methods, but one must resort to the bromine pyridine method with subsequent iodometric titration of the bromine.¹⁹ The hydrogenation of the double bond of cholesterol leads to cholestanol (XVI) and epicholestanol (XVII), depending on the catalyst used. The $>\text{CHOH}$ of both these saturated alcohols can be oxidized yielding the identical saturated ketone, cholestanone, which in turn is reduced by Clemmensen's method to the saturated hydrocarbon cholestane (XVIII).



Coprostanol (Coprosterol).—This substance, first discovered by Austin Flint in feces and called stercorin, was subsequently rediscovered after it had been forgotten for thirty-five years.²⁰ It is a stereoisomer of cholestanol and was reputed to be the reduction product of cholesterol by the intestinal flora; this bio-reduction could, however, never be verified *in vitro*. Coprostanol differs from cholestanol by the configuration on C₅. Coprostanol can be obtained *in vitro* by hydrogenation of coprostenol (allocholesterol of the older literature). This substance is a structural isomer of cholesterol; its double bond lies between C₄ and C₅, but slips easily, *e. g.*, under the influence of weak acid into the position C₅=C₆, forming ordinary cholesterol. But when H-H is added to its double bond in C₄=C₅, the *cis* configuration of ring I relative to ring II follows, as can be seen by inspection of a spatial model. Thus, coprostenol is presumably the physiologic precursor of coprostanol and also of the bile acids where the same steric configuration prevails. The saturated hydrocarbon derived from coprostanol, *via* the ketone coprostanone, is called coprostane (pseudocholestane). This substance and cholic acid (VIIIa) can be transformed into each other, parallel to the formation of allocholic acid from cholestane (XVIII, see p. 140).

Cholesterol seems to be subject to both hydrogenation and oxidation in the animal body. Its dihydroderivative, cholestanol, is found in sterile intestinal cysts,²¹ and in small concentrations in the blood serum. It is formed in the body and excreted together with cholesterol¹³ into the intestinal lumen, whence it cannot be resorbed again. The presence of "oxycholesterol" (XIX), a glycol or dihydroxyderivative, in blood serum, has long been suspected. It has been prepared in the crystalline state by Rosenheim and King,²² and was found to rearrange easily with loss of one H₂O, into the unsaturated coprostenone (cholestenone, XX) which can be reduced to coprostenol (XXI) and further hydrogenated (by the intestinal flora) to coprostanol (XXII). This sequence of reactions may well be the method used by the animal organism to accomplish a transition from the cholestane-cholesterol series into the coprostanone-bile acid series.

The physiologic functions of cholesterol are little understood. The cholesterol level of the blood is subject to considerable physiologic fluctuations during the twenty-four hours of the day in connection with the digestive processes and especially due to the excretion of cholesterol in the intestinal juice. Pathologic hypercholesteremia is familiar in biliary obstruction. Severe changes of the plasma proteins such as in nephrosis, anemia and other conditions, characterized by low blood protein content, also experimental anemia produced by repeated withdrawal of blood or plasma (plasmaphoresis) are as a rule accompanied by the release of huge amounts of cholesterol into the circulation, and values as high as 2000 mg. per 100 cc., *i. e.*, 2 per cent, as found in nephrosis, surpass the highest values of hypercholesteremia in biliary disease. In all these conditions, hypercholesteremia is accompanied by

general lipemia and the typical opacity of the serum. High cholesterol values in serum are also common in diabetes mellitus, and in xanthomatoses. Xanthelasmata, as found on the eyelids, consist of cholesterol plus lipochromes; a general deposition of cholesterol, especially its esters, together with lipochromes in necrotized bone areas, chiefly in the skull, is the characteristic feature of Schüller-Christian's disease. In another lipid storage disease, Niemann-Pick's disease, the accumulation of phosphatides in liver, spleen, bone marrow, etc., is also accompanied by an increase of cholesterol and its esters.²³ The most common pathologic condition involving cholesterol storage is atherosclerosis where large amounts of it are deposited in the intima of the arteries. This can be reproduced experimentally, as in rabbits, by cholesterol feeding, where the cholesterol deposits may even involve the veins.

Fatty acids and their salts, the soaps, are unsuitable for use as transport forms for the important fuel fat. On the other side, the physical properties of neutral fat render it unsuitable for this purpose, since it usually does not assume the finely divided form necessary for transport and distribution through cellular structures. Cholesterol esters and phosphatides with their peculiar colloidal properties offer possibilities for the transport of fatty acids and allow the deposition of neutral fat at the point of destination. Thus, cholesterol seems to function as an important vehicle in lipid metabolism.

The ratio, free cholesterol:combined cholesterol in plasma or serum—red blood corpuscles contain free cholesterol only—is usually given as "ester percentage" = combined cholesterol/total cholesterol. The values of this fraction normally oscillate between 40 and 70 per cent. This proportion is regulated by the liver and certain diseases of this organ disturb the ratio in the sense of diminished ester percentage.²⁴

Other sterols occurring in the animal kingdom are lanosterol, $C_{30}H_{50}O$, melting point = $141^{\circ}C.$, and agnosterol, $C_{30}H_{48}O$, melting point = $162^{\circ}C.$, in wool fat (lanolin), bombycesterol in the larvae of the silkworm (*Bombyx mori*), spongosterol in sponges, asteriasterol in the eggs of the starfish, and ostrasterol in bivalves.²⁵ Sterols typical for the lower plants are ergosterol, $C_{28}H_{44}O$, melting point = $160^{\circ}C.$ and zymosterol, melting point = $109^{\circ}C.$, both occurring in yeast. Ergosterol was found first in ergot²⁶ which is a higher fungus; it was also isolated from molds (*mucor*). Bacteria probably contain no sterols.

Ergosterol.—Ever since this sterol was recognized to be "provitamin D," its chemistry has been intensively studied. Ergosterol contains the same carbon skeleton as cholesterol, but it differs from the latter by possessing one more methyl group on C_{24} in the side-chain and three double bonds, one of which is situated in the side-chain $C_{22}=C_{23}$. The hydroxyl group is on C_3 as in cholesterol. Ultraviolet irradiation of various wave lengths causes shifts in the position of the double bonds and in the steric configuration.²⁷ Thus a number of iso-

mers are formed, all of which respond to the formula $C_{28}H_{44}O$ and carry the names lumisterol, protachysterol, tachysterol, vitamin D (calciferol), suprasterol I and suprasterol II. These products of isomerization by ultraviolet rays have not as yet been reproduced by purely chemical methods.

Phytosterols.—The most important and best known representatives of the phytosterols, the sterols of higher plants, are **sitosterol**, $C_{29}H_{50}O$, melting point = $137^{\circ}C$. (Greek *σιτος* = wheat), and **stigmasterol**, $C_{29}H_{48}O$, melting point = $170^{\circ}C$. The unsaponifiable fraction of some plant fats contains mainly sitosterol (wheat and barley germs, linseed oil, cotton oil, laurel oil), while in others varying amounts of the doubly unsaturated stigmasterol are found (as in the calabar bean, in beet oil, coconut oil, and cocoa butter). The phytosterols may be accompanied by the closely related saponins, lupeol, amyryne, and their esters. One double bond of stigmasterol is located in the side-chain in the same position as in ergosterol $C_{22}=C_{23}$. C_{24} carries an ethyl group instead of a methyl group, as in ergosterol. The other double bond of stigmasterol as well as the one in sitosterol are in the same position ($C_5=C_6$) as in cholesterol. All the sterols mentioned, with the exception of the sterols of wool fat and of the irradiation products of ergosterol, can be precipitated with digitonin. The rôle of the phytosterols in plant metabolism remains to be elucidated. As they cannot be resorbed in significant amounts through the intestinal wall of the usual laboratory animals, they have probably no significance for the sterol metabolism of man and higher animals.²⁸

Physiologic Action of Bile Acids, Choleic Acids.—However indefinite our knowledge is in regard to the functions of the sterols, their analytical estimation, especially that of cholesterol, is both easy and accurate, and cholesterol determinations in blood or plasma have become a valuable routine procedure in clinical chemistry. The converse holds for the bile acids, where we deal with a group of substances whose properties furnish a plausible basis for the interpretation of their function, but clinical study of which is hampered by the lack of convenient analytical methods.

The constitution and terminology of the bile acids has been outlined in the beginning of this chapter. Their biochemical significance is founded on their high surface activity and their emulsifying power for fats. But, beyond these physical properties, the ability of certain bile acids to form chemical "coordination" or "addition compounds" with a great many types of substances enhances their physiologic importance. Choleic acid, isolated from animal biles, consists of about 8 per cent fatty acid and 92 per cent deoxycholic acid. This led to the discovery of the "choleic acid principle." A great many substances such as fatty acids, alcohols, phenols, ketones (*e. g.*, camphor), hydrocarbons (*e. g.*, naphthalene) combine with deoxycholic acid in the molecular ratios 1:2, 1:3, 1:4, 1:6, or 1:8, depending

on size and shape of the molecule. The resulting substances, termed "choleic acids"—for instance: Stearic acid-choleic acid, phenol-choleic acid, camphor-choleic acid—are coordination or molecular compounds, held together by "secondary" valencies.²⁹ Since their alkali salts are water-soluble, many water-insoluble substances can be rendered soluble through choleic acid formation and can then permeate structures of the animal body, otherwise inaccessible.

While this peculiarity of deoxycholic acid is shared only to a limited extent by other bile acids, its significance for the theory of intestinal resorption is apparent. While the physical process of emulsification cannot be ignored, any explanation of lipid resorption and transport would be incomplete without inclusion of the choleic acid principle. Bile acids not only promote the diffusion of lipids from the intestinal tract into the lymph spaces of the villi and hence into the lymph ducts and the portal system, but also exert a favorable influence on the action of the pancreatic lipase within the intestine. Physiologists still differ in the relative appreciation of the significance of these factors for the proper functioning of fat resorption.

Aside from the resorption of this important fuel material, the bile acids also influence the intestinal resorption of many regulatory substances such as the lipid soluble members of the vitamin group, hormones, drugs and other physiologically active substances. *E. g.*, female sex hormone, due to its lipid character, has a definite affinity toward bile; it has been detected in the gallbladder contents of an old man, and its ability to form a choleic acid was put to preparative use.³⁰

The formation of choleic acids is perhaps responsible for the resorption of alkaloids like strychnine, which would otherwise remain insoluble and not resorbable from the alkaline intestinal content. These properties of the bile acids have been used advantageously for therapeutic measures. The sodium salt of the triketo derivative, dehydrocholic acid, has been applied in combination with various pharmaceutical preparations in order to enhance and accelerate their resorption. Lipolytic dyes, containing a high percentage of iodine, are used for the visualization of the gallbladder, and lipolytic organic mercury compounds find a place among disinfectants which are directed by their bile-affinity specifically into the gallbladder.

Differences of tendency to form choleic acids have been observed between the dextro-form and the levo-form of the same chemical compound. Thus, the bile acids may be one of the tools which help the organism in selecting and differentiating a physiologically useful optically active compound from its nonphysiologic mirror-image. Furthermore, deoxycholic acid has an enolizing influence on tautomeric substances, *i. e.*, compounds consisting of a mixture of two forms, namely, a "keto" form and a more reactive unsaturated or "enol" form. Ethyl aceto-acetate for instance, when forming a choleic acid with deoxycholic acid, is completely converted into the enol form. This may be

of significance in the utilization of fatty acids by *beta*-oxidation through the intermediary formation of enolizable β -keto acids.³¹

This enolizing influence of bile acids has been adduced to explain the difference between the "direct" and "indirect" diazo reaction of bilirubin of Hymans van den Bergh. Bilirubin, in the serum of jaundiced subjects, seems to occur in two forms: One form, surmised to be the enol, couples readily with diazotized sulfanilic acid (Ehrlich's reagent), ("direct reaction"), while the other one reacts only after it has been digested with alcohol ("indirect"). The simultaneous presence of bile acids, as in obstructive and "hepatic" jaundice, is prerequisite for the "direct" reaction, while bilirubin of extrahepatic (hemolytic) origin occurs in the keto form and requires the alcohol treatment for enolization ("indirect reaction" only).³¹

Cholesterol, excreted through the bile, is kept in solution by the great excess of bile acids in this fluid. The stability of its solution or, on the other hand, its tendency to precipitate and to form gall stones depends (1) on the ratio cholesterol/bile acids in the bile, (2) the pH of the bile (see next paragraph), (3) on the qualitative nature of the bile acids present. While less is known about this third point, it has been observed in regard to the first point, that the bile of human gall-bladders in which stones are found at autopsy, regularly show a ratio bile acids: cholesterol of less than 8: 1, while stone-free bile contains higher relative amounts of bile acids, regardless of the absolute amounts of solid ingredients in either group.³²

The pH of Bile.—Bile, as excreted from the liver, displays a slightly alkaline reaction 7.8 and 8.6. This pH is remarkably stable and hardly responds to extreme changes in the acidity or alkalinity of the diet. Bile contains 1 to 4 per cent solid constituents, the bile acids in the form of their sodium salts, fatty acids, minor amounts of cholesterol and lecithin, inorganic constituents of the same type as in blood serum, with sodium chloride prevailing, carbon dioxide, urea, and fluctuating amounts of mucin. Gallbladder bile may contain as much as 20 per cent total solids as a result of a ten-fold concentration within a few hours.³³

It seems paradoxical, then, that both liver bile and gallbladder bile should be isotonic with blood serum and the other body fluids. But this fact has been ascertained by freezing point determinations and can be explained by the gradual diminution of the inorganic constituents through diffusion from 0.8 per cent to 0.2 per cent, while the relative and absolute quantity of organic components increases.³⁴ These conditions must be governed by Donnan's equilibrium, as we are dealing with a mixture of two salts with an identical cation but anions of differing diffusibility, namely, of sodium chloride plus sodium bile salts within the gallbladder, and a solution of sodium chloride in the outside fluid, the blood in the capillary system of the bladder wall. Although the constant flow of blood and the periodic emptying of the gallbladder prevents the establishment of a true equilibrium, the gallbladder bile

turns more acid in accordance with Donnan's law and reaches pH-values as low as 6 in the concentrated state.

Among the physiologic and pharmacological effects of the bile acids, one should enumerate their retarding action on the heart.³⁵ Their cathartic action which contributes to the smooth functioning of intestinal digestion is used therapeutically in bile salt preparations and in bile enemas. The bitterness of bile acids has been utilized for measuring the blood circulation time, as the bitter taste appears sharply in a given time after intravenous injection. Bile and bile acids have a lytic power for pneumococci and red blood corpuscles. They increase the viscosity of the blood plasma. Their high surface activity can be used for their detection and estimation in urine. The best known application of this principle is the *Hay test*. If flowers of sulfur do not float on the surface of the urine to be tested, its surface tension is depressed at least as much as by 0.05 per cent glycocholic acid; if the sulfur floats, then the concentration of bile acid is below this figure.

Analytical Methods.—The most widely used qualitative test for bile acids is Pettenkofer's reaction,³⁶ a purple coloration on addition of sugar and concentrated sulfuric acid. This reaction has been carried out in numerous modifications using rhamnose, furfural, vanillin and other compounds instead of sugar, and phosphoric acid or 70 per cent sulfuric acid instead of the concentrated acid. Its application in quantitative analysis has never been generally accepted. However, the modification with 70 per cent sulfuric acid and furfural (blue color) which is given by cholic acid only, but not by the *di*-hydroxy acids, as deoxycholic acid, serves as a useful colorimetric procedure.³⁷ A quantitative estimation comprising the entire group of bile acids can be based on their isolation as ferric salts and subsequent estimation of the iron. The conjugated bile acids can be determined by estimation of the amino-nitrogen in van Slyke's apparatus before and after saponification (deconjugation), and the percentage of tauro-acids by a simultaneous sulfur determination.³⁸

The amounts found in urine under pathologic conditions may reach 50–100 mg. per 100 cc. The determination in blood and serum meets with many obstacles; 20–30 mg. per 100 cc. will be the upper limit in the blood during acute biliary obstruction.

While the hepatic origin of the bile acids is not disputed, there is no direct evidence that they are derived from cholesterol, but chemical considerations favor this hypothesis. The cyclic portion of the carbon skeleton in the cholesterol-allocholanolic series (which also includes the phytosterols) on one hand, and in the coprostanol-cholanolic acid series on the other, are identical except for the steric configuration on C₅. A glance at the formula of Rosenheim and King for cholanolic acid reveals eight asymmetric carbon atoms, namely, 5 and 10, 8 and 9, 13 and 14, 17 and 20.

It then seems remarkable that sterols and bile acids should show similar configurations except for the situation of a single carbon

atom, although $2^8 = 256$ stereomeric configurations are theoretically possible. This peculiarity reminds one of the unique prevalence of *d*-glucose in nature among the numerous possible stereomeric aldohexoses. The tetracyclic carbon skeleton of the sterols and the bile acids has earned its widespread occurrence in nature by its great stability. This skeleton is hardly attacked by micro-organisms and will persist in a carcass as "adipocire" long after carbohydrates, lipids and proteins have fallen a prey to the various saprophytes. No fragments that would offer a clue to the manner of its catabolism have been found in the living or dead animal body nor in its excretion products.

HARRY SOBOTKA.

REFERENCES

1. Mauthner, L.: *Monatsh.*, 15-30 (1894-1909); Diels, O.: *Ber.*, 36-45 (1903-1912); Windaus, A.: *Ber.*, 36 to date; Wieland, H.: *Z. physiol. Chem.*, 80 to date.
2. Rosenheim, O., and King, H.: *Chemistry Industry*, 51, 464 (1933).
3. Rosenheim, O., and King, H.: *Annual Review Biochem.*, 3, 87 (1934); Sobotka, H.: *Chem. Reviews*, 15, No. 3 (1934).
4. Hammersten, O.: *Z. physiol. Chem.*, 24, 322 (1898); Tschesche, R.: *Z. physiol. Chem.*, 203, 263 (1932).
5. Wieland, H., and Alles, R.: *Ber.*, 55, 1789 (1922).
6. Marrian, G. F.: *Science Progress*, 1933; Girard, A.: *Bull. soc. chim. biol.*, 15, 562 (1933).
7. Diels, O., et al.: *Ber.*, 60, 140 (1927); Rosenheim, O., and King, H.: *Chemistry Industry*, 52, 299 (1933).
8. Lachinov, P.: *Ber.*, 12, 1526 (1879).
9. Cook, J. W., Dodds, E. C., and Hewett, C. L.: *Nature*, 131, 56, 205 (1933).
10. Kennaway, E. L., and Cook, J. W.: *Chemistry Industry*, 51, 521 (1932).
11. Chevreul, M.: *Ann. chim.*, 95, 5 (1815).
12. Couerbe, J. P.: *Ann. chim. phys.*, (ii), 56, 160 (1834).
13. Sperry, W. M.: *J. Biol. Chem.*, 71, 351 (1927); 96, 769 (1932); Schönheimer, R., et al.: *Z. physiol. Chem.*, 192, 73 (1930).
14. Bernouilli, A. L.: *Helv. chim. Acta*, 15, 2741 (1932).
15. Bloor, W. R.: *J. Biol. Chem.*, 29, 437 (1917).
16. Windaus, A.: *Ber.*, 42, 238 (1909).
17. Berthelot, A.: *Ann. chim.*, 56, 51 (1859).
18. Huerthle, K.: *Z. physiol. Chem.*, 21, 331 (1896).
19. Page, I. H., Pasternak, L., and Burt, M. L.: *Biochem. Z.*, 223, 445 (1930).
20. Flint, A., Jr.: *Amer. J. Med. Sci.*, 44, 305 (1862); Bondzynski, St., and Humnicky, V.: *Z. physiol. Chem.*, 22, 396 (1897).
21. Boehm, R.: *Biochem. Z.*, 33, 477 (1911); Schönheimer, R., and Hrdina, L.: *Z. physiol. Chem.*, 212, 161 (1932).
22. Rosenheim, O., and King, H.: *Chemistry Industry*, 52, 1056 (1933).
23. Sobotka, H., et al.: *Biochem. J.*, 27, 2031 (1933).
24. Thannhauser, S. J., and Schaber, F.: *Klin. Wochschr.*, 5, 252 (1926); Epstein, E. Z.: *Arch. Internal Med.*, 47, 82 (1931); 50, 203 (1932).
25. Windaus, A., and Tschesche, R.: *Z. physiol. Chem.*, 190, 51 (1930); Menozzi, A., and Moreschi, R.: *Rc. Ac. Lincei* (5), 17, 1, 95 (1908); Henze, M.: *Z. physiol. Chem.*, 41, 109 (1904); 55, 427 (1908); Page, I. H.: *J. Biol. Chem.*, 57, 471 (1923); Bergmann, W.: *J. Biol. Chem.*, 104, 317, 553, (1934).
26. Tanret, C.: *Compt. rend.*, 147, 75 (1908).
27. Rosenheim, O., and King, H.: *Chemistry Industry*, 53, 196 (1934).
28. Anderson, R. J., et al.: *J. Am. Chem. Soc.*, 46 and 48 (1924 and 1926); Sandqvist, H., et al.: *Ber.*, 63, 1935 (1930); 64, 2167 (1931); Schönheimer, R.: *Z. physiol. Chem.*, 185, 119 (1929).
29. Wieland, H., and Sorge, H.: *Z. physiol. Chem.*, 96, 1 (1916); Rheinboldt, H., et al.: *Ann.*, 451, 256 (1926); 473, 249 (1929); *Z. physiol. Chem.*, 180, 180 (1928); 184, 219 (1929); Sobotka, H., and Goldberg, A.: *Biochem. J.*, 26, 555, 905 (1932).

30. Wieland, H., Straub, H., and Dorfmueller, Th.: *Z. physiol. Chem.*, **186**, 97 (1930).
31. Sobotka, H., and Kahn, J.: *Biochem. J.*, **26**, 898 (1932); *Ber.*, **65**, 227 (1932); Fowweather, F. S.: *Biochem. J.*, **26**, 165 (1932).
32. Newman, C. E.: *Ziegler's Beitr. Path.*, **86**, 187 (1931); Andrews, E., Schönhaimer, R., and Hrdina, L.: *Arch. Surgery*, **25**, 796 (1932).
33. Rous, P., and McMaster, P. D.: *J. Exp. Med.*, **34**, 47 (1921).
34. Brand, J.: *Arch. ges. Physiol.*, **90**, 491 (1902); Ravdin, I. S., *et al.*: *Am. J. Physiol.*, **100**, 317 (1932).
35. Wieland, H., and Hildebrand, Th.: *Arch. exp. Path. Pharmacol.*, **85**, 199; **86**, 79, 92 (1920).
36. Pettenkofer, M.: *Ann.*, **52**, 90 (1844).
37. Gregory, R., and Pascoe, P. A.: *J. Biol. Chem.*, **83**, 35 (1929); Reinhold, J. G., and Wilson, D. W.: *J. Biol. Chem.*, **96**, 637 (1931).
38. Foster, M. G., and Hooper, C. W.: *J. Biol. Chem.*, **38**, 355 (1919); Schmidt, C. L. A., and Dart, A. E.: *J. Biol. Chem.*, **45**, 415 (1921).

CHAPTER V

THE PROTEINS

OUR information on the composition of proteins is derived from the study of their products of decomposition by the process of hydrolysis. The result of hydrolysis is a complex mixture of amino acids which requires separation into individual substances with their characterization and identification. The separation was first attempted by direct crystallization of the amino acids, or of their copper, silver or other salts. It was accomplished only if an amino acid were present in large amount. Though an amino acid might thus be isolated from one or another protein, it was not proved that it was present in most or all proteins. Several amino acids were meanwhile isolated from extracts of vegetable and animal tissues and were only later recognized as constituents of proteins. Advances were made only by the employment of new methods of separation. To Drechsel and especially to Kossel and his pupils, we owe our chief knowledge of the three basic amino acids, which have been found in all proteins. To Emil Fischer and his school we owe the so-called "ester" method of separating the simple monoamino acids, which proved their presence in most proteins and led to the discovery of two additional amino acids. To Hopkins and Cole we owe tryptophan, the presence of which in proteins was known only by its special color reactions. The first amino acids isolated from proteins were leucine and glycine in 1820. The last was methionine in 1922. The order of discovery of the amino acids is given in the following table. In this table are given only those amino acids which are definitely present in most if not all proteins. Vickery and Schmidt (1931) consider that only those amino acids should be included which have been found in more than one protein and by more than one investigator.

DISCOVERIES OF THE AMINO ACIDS

| <i>Date.</i> | <i>Amino acid.</i> | <i>Discovered by</i> | <i>First isolated by</i> | <i>In</i> | <i>From.</i> |
|--------------|--------------------|----------------------|--------------------------|-----------|-----------------------------|
| 1820 | Leucine | Braconnot | Proust | 1819 | extract of fermented gluten |
| 1820 | Glycine | Braconnot | | | |
| 1849 | Tyrosine | Bopp | Liebig | 1846 | cheese |
| 1865 | Serine | Cramer | | | |
| 1866 | Glutamic acid | Ritthausen | | | |
| 1868 | Aspartic acid | Ritthausen | Plisson | 1827 | asparagine |
| 1879 | Alanine | ?Schutzenberger | | | |
| 1888 | Alanine | Weyl | Strecker | 1850 | by synthesis |
| 1883 | Phenylalanine | Schulze and Barbieri | Schulze and Barbieri | 1879 | sprouts of lupines |
| 1889 | Lysine | Drechsel | | | |
| 1896 | Arginine | Hedin | Schulze and Steiger | 1886 | sprouts of lupines |
| 1896 | Histidine | Hedin | | | |
| | | Kossel | | | |
| 1899 | Cystine | K. A. H. Mörner | Wollaston | 1810 | urinary calculus |
| 1901 | Valine | E. Fischer | v. Gorup-Besanez | 1856 | extract of pancreas |
| 1901 | Proline | E. Fischer | Willstätter | 1900 | by synthesis |

DISCOVERIES OF THE AMINO ACIDS, *Continued*

| Date. | Amino acid. | Discovered by | First isolated by | In | From. |
|--|--------------------------|---|-----------------------------|------|---------------|
| 1901 | Tryptophan | Hopkins and Cole | | | |
| 1902 | Hydroxyproline | E. Fischer | | | |
| 1903 | Isoleucine | F. Ehrlich | F. Ehrlich | 1903 | beet molasses |
| 1918 | Hydroxyglutamic acid | Dakin | | | |
| 1922 | Methionine | Mueller | | | |
| To these must be added the following which are found only in special proteins: | | | | | |
| Date. | Amino acid. | Discovered by | | | |
| 1896 | Iodogorgoic acid | Dreschsel | in skeleton of sponges | | |
| 1929 | Iodogorgoic acid | Harrington and Randall | in thyroid | | |
| 1913 | Dibromotyrosine | C. T. Mörner | in horny skeleton of corals | | |
| 1901 | Norleucine | Thudichum | | | |
| 1913 | Norleucine | Abderhalden and Weil | in brain | | |
| 1915 | Thyroxine | Kendall | in thyroid protein | | |
| Several other amino acids have been described, namely: | | | | | |
| | Hydroxyaminobutyric acid | by Schryver and Buston in vegetable proteins | | | |
| | Hydroxyvaline | | | | |
| | Hydroxylysine | | | | |
| | Protocetine | | | | |
| | Dihydroxyphenylalanine | by Guggenheim in leguminous plants | | | |
| | Citrulline | by Wada in melon juice, 1930; in casein, 1933 | | | |

The Constitution of the Amino Acids.—The amino acids have been proved to have the following formulae:

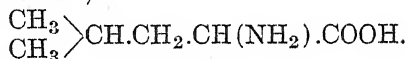
I. Simple monoamino acids:

Glycine, or amino-acetic acid, $\text{CH}_2(\text{NH}_2).\text{COOH}$.

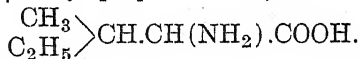
Alanine, or α -amino-propionic acid, $\text{CH}_3.\text{CH}(\text{NH}_2).\text{COOH}$.

Valine, or α -amino-isovaleric acid, $\begin{matrix} \text{CH}_3 \\ \text{CH}_3 \end{matrix} \text{CH}(\text{CH}_3).\text{CH}(\text{NH}_2).\text{COOH}$.

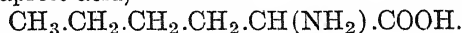
Leucine, or α -amino-isocaproic acid,



Isoleucine, or α -amino- β -methyl- β -ethyl-propionic acid,

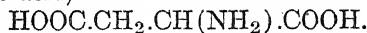


Norleucine, or α -aminocaproic acid,

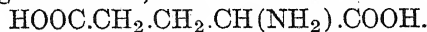


II. Amino dicarboxylic acids:

Aspartic acid, or α -aminosuccinic acid,

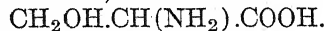


Glutamic acid, or α -aminoglutaric acid,

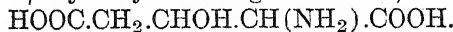


III. Hydroxy-amino acids:

Serine, or β -hydroxy- α -amino-propionic acid,

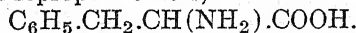


Hydroxyglutamic acid, or β -hydroxy- α -aminoglutaric acid,

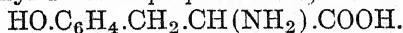


IV. Amino acids with aromatic nuclei:

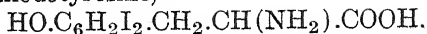
Phenylalanine, or β -phenyl- α -aminopropionic acid,



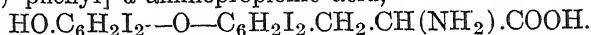
Tyrosine, or β -*p*-hydroxyphenyl- α -amino-propionic acid,



Iodogorgoic acid, or 3, 5-diiodotyrosine,

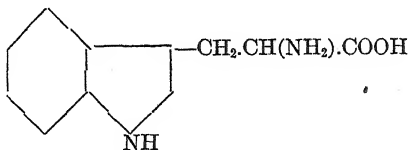


Thyroxine, or β -[3, 5-diiodo-4-(3', 5'-diiodo-4'-hydroxyphenoxy) phenyl]- α -aminopropionic acid,



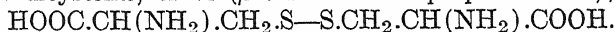
V. Amino acid with indole nucleus:

Tryptophan, or β -indole- α -amino-propionic acid,

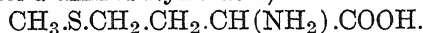


VI. Amino acids containing sulfur:

Cystine, or dicysteine, or di-(β -thiol- α -amino-propionic acid),



Methionine, or γ -methylthiol- α -aminobutyric acid,

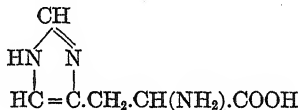


VII. Basic amino acids, or hexone bases:

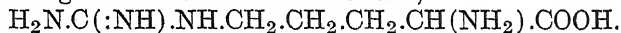
Lysine, or α , ϵ -di-aminocaproic acid,



Histidine, or β -imidazole- α -amino-propionic acid,

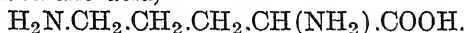


Arginine, or δ -guanidine- α -amino-valeric acid,

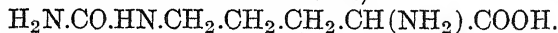


To which must be added:

Ornithine, or α , δ -diaminovaleric acid,

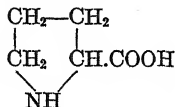


Citrulline, or δ -carbamido- α -amino-valeric acid,

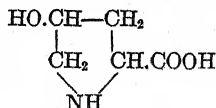


VIII. Amino acids with pyrrole nuclei:

Proline, or α -pyrrolidine carboxylic acid,



Hydroxyproline, or γ -hydroxy- α -pyrrolidine carboxylic acid,



The determination of the constitution of the amino acids in the early days was a difficult problem, and often many years elapsed between the discovery of an amino acid and the proof of its structure by synthesis. With improved methods of synthesis of an unknown amino acid is now readily synthesized.

All the amino acids except glycine are optically active, and only one stereo-isomer exists in the protein molecule. In the cases of cystine, hydroxyglutamic acid and hydroxyproline, with two asymmetric carbon atoms, four stereo-isomers are possible. The synthesis of an amino acid is complete only when the optical isomers have been separated. A few of the synthetic amino acids still require separation into their stereo-isomers. The following table shows the dates and authors of the first syntheses of the amino acids and also the natural form and its separation from the racemic mixture:

DATES OF SYNTHESSES OF THE AMINO ACIDS AND THEIR NATURAL FORMS

| | <i>Synthesized by</i> | <i>In</i> | <i>Natural form</i> | <i>Form separated by</i> | <i>In</i> |
|----------------------|---------------------------|-----------|-------------------------|------------------------------|-----------|
| Glycine | Perkin and Duppa | 1858 | | | |
| Alanine | Strecker | 1850 | d- | E. Fischer | 1899 |
| Valine | Fittig and Clark | 1866 | d- | E. Fischer | 1906 |
| Leucine | Limpricht | 1855 | l- | E. Fischer | 1900 |
| Isoleucine | Bouveault and Locquin | 1905 | d- | Locquin | 1907 |
| Norleucine | Schulze and Likiernik | 1893 | d- | E. Fischer and Hagenbach | 1901 |
| Aspartic acid | Dessaignes | 1850 | l- | Piutti | 1887 |
| Glutamic acid | Wolf | 1890 | d- | E. Fischer | 1899 |
| Serine | E. Fischer and Leuchs | 1902 | l- | E. Fischer and Jacobs | 1906 |
| Hydroxyglutamic acid | Dakin | 1919 | d- | | |
| Phenylalanine | Erlenmeyer and Lipp | 1883 | l- | E. Fischer and Scholler | 1907 |
| Tyrosine | Erlenmeyer and Lipp | 1883 | l- | E. Fischer | 1900 |
| Iodogorgoic acid | Wheeler and Jamieson | 1905 | | | |
| Thyroxine | Harrington and Barger | 1927 | l- | | |
| Tryptophan | Ellinger and Flamand | 1907 | l- | | |
| Cystine | Erlenmeyer | 1903 | l- | E. Fischer and Raske | 1908 |
| Methionine | Barger and Coyne | 1928 | l- | Windus and Marvel | 1931 |
| Lysine | E. Fischer and Weigert | 1902 | d- | | |
| Histidine | Pyman | 1911 | l- | Pyman | 1911 |
| Arginine | Schulze and Winterstein | 1899 | d- | | |
| Proline | Willstätter | 1900 | l- | E. Fischer and Zemplén | 1909 |
| Hydroxyproline | Leuchs | 1905 | l- | | |

Hydrolysis of Proteins.—Hydrolysis of proteins to the amino acids is effected by boiling with acids, or alkalis, or by the action of the enzyme trypsin. The choice of hydrolyzing agent depends largely upon the products that it is desired to isolate. The isolation of all the amino acids has not often been carried out with one lot of material.

Hydrolysis with sulfuric acid is preferred as the acid can be subsequently removed with barium hydroxide. It is carried out by boiling the protein with six times its quantity of 25 or 33 per cent sulfuric acid for fifteen to twenty-four hours. It is employed when the isolation of the basic amino acids or a butyl alcohol extraction is required. Hydrolysis with hydrochloric acid is preferred when the monoamino acids are required. It is carried out by boiling the protein with three times the quantity of conc. HCl, or with six times the quantity of 20 per cent HCl for six to twenty-four hours. Complete hydrolysis is also effected by dissolving the protein in 3N acid and then heating for one and one half to two hours in an autoclave at 150°.

The completion of the hydrolysis is determined by carrying out the

biuret reaction which must be negative. It is better to ascertain completion by determination of amino nitrogen by Van Slyke's method at intervals of five to six hours.

Hydrolysis with alkali is seldom performed, since alkali destroys arginine and cystine and racemizes the other amino acids. It is carried out for the estimation of tryptophan. Hydrolysis with trypsin is used for the isolation of tyrosine, tryptophan, and methionine.

Isolation of the Amino Acids.—Full details of the procedure for the isolation of the several amino acids cannot be described in this chapter. The following outline will indicate the series of manipulations.

Isolation of Cystine and Tyrosine.—Cystine and tyrosine are very slightly soluble in water, but their solubility is influenced by the presence of other amino acids in the solution. They are not, therefore, completely precipitated on neutralizing an acid solution after hydrolysis. Generally the amount of cystine in proteins is quite small and this amino acid does not separate out. Only the keratin group of proteins usually yields cystine on neutralizing the solution. The solution is nearly neutralized and finally made neutral to Congo red with sodium acetate. In the case of tryptic digest, a large part of the tyrosine generally separates out during the course of the digestion.

Isolation of Methionine.—Pirie⁶¹ has described a simple way of preparing methionine from casein and other proteins. The protein is digested with trypsin and the filtered solution is precipitated with mercuric acetate in acetic acid solution with the addition of some phosphotungstic acid.

Isolation of Tryptophan.—The isolation of tryptophan depends upon its precipitation with mercuric sulfate in sulfuric acid solution after trypsin digestion of the protein. It is subsequently separated from histidine and cystine. The separation is readily effected by butyl alcohol extraction.

Extraction with Butyl Alcohol.—This method described by Dakin¹⁸ may serve for the separation of the series of amino acids into four groups. The solution of the amino acids is exactly neutralized and extracted with butyl alcohol, most conveniently in a continuous extractor, for fifty to one hundred hours depending on the rate of butyl alcohol distillation. With casein the method gives a very good separation, but with other proteins the results are not so good. The butyl alcohol containing water dissolves the simple monoamino acids together with proline. On evaporation of the butyl alcohol, the residue is extracted with absolute alcohol, which dissolves proline. The other amino acids require separation by the ester method. Butyl alcohol extraction is most useful for the isolation of proline. The other amino acids are not generally completely extracted. The portion not dissolved by the butyl alcohol consists mainly of the basic amino acids and the dicarboxylic acids. The basic amino acids are separated by precipitation with phosphotungstic acid, and the dicarboxylic acids are thrown down as barium salts with alcohol.

The Ester Method of Separating the Monoamino Acids.—For this purpose the protein is hydrolyzed with hydrochloric acid. The solution is concentrated and saturated with hydrogen chloride so as to separate a portion of the glutamic acid as hydrochloride. The solution is evaporated to a syrup *in vacuo* so as to remove as much water as possible. The pasty residue is dissolved in absolute alcohol and the alcohol is saturated with hydrogen chloride, so as to convert the amino acids into their esters. The alcohol is removed by evaporation *in vacuo* and the esterification process is repeated. Of the ester hydrochlorides so obtained, glycine ester hydrochloride is soluble with difficulty, and if this amino acid is present in large amount, it crystallizes out. The solution is concentrated *in vacuo*, and the esters are liberated from their hydrochlorides by treatment with solid baryta and extraction with ether. The ethereal solution is dried, the ether distilled off and the oily residue of esters fractionally distilled *in vacuo*. The various fractions of esters are saponified by boiling with water, or by means of baryta. The amino acids contained in the fractions are separated by crystallization.

The fraction containing valine, leucine and isoleucine is very difficult to separate into its constituents, and the yield of these monoamino acids generally represents a mixture mostly of leucine and isoleucine.

The Separation of the Basic or Diamino Acids.—These diamino acids are separated after hydrolysis with sulfuric acid. More recently they have been isolated after hydrolysis with hydrochloric acid, which is removed with silver oxide and sulfuric acid. Histidine is precipitated by adding excess of silver as oxide in presence of sulfuric acid and making the solution neutral to pH 7 with barium hydroxide. Arginine is then precipitated by making the solution alkaline to phenolphthalein (pH 10). From the filtrate the silver is removed and the lysine precipitated by phosphotungstic acid in acid solution. This procedure was first described by Kossel and Patten⁵³ and has been improved and perfected by Vickery, Leavenworth and Block,^{75, 76} who consider the procedure the best way of isolating these diamino acids. Cystine may contaminate the histidine but is separated as the copper salt. The histidine and arginine are finally obtained as flavianates, and lysine as picrate. From these salts, histidine chloride and arginine carbonate may be obtained. This method of isolation of the basic amino acids is regarded as quantitative. Arginine may be directly precipitated from the hydrolyzed solution of the protein as flavianate and in this way may be easily prepared from gelatin.⁶³

If the diamino acids are precipitated first as phosphotungstates, after a butyl alcohol extraction, the compound is decomposed with baryta after solution in 50 per cent acetone and the above separation of the silver salts is carried out.

The following table shows the amounts of amino acids which have been isolated from a selected list of proteins:

AMOUNTS OF AMINO ACIDS ISOLATED FROM PROTEINS

| | Ovalbumin. | Lactalbumin. | Casein. | Vitelin. | Hemoglobin. | Gelatin. | Silk fibroin. | Wool keratin. | Salmin. | Wheat gliadin. | Wheat glutenin. | Edestin (Hemp) | Legumin (Pea) | Amandin (Almond). |
|----------------------------|------------|--------------|---------|----------|-------------|----------|---------------|---------------|---------|----------------|-----------------|----------------|---------------|-------------------|
| Glycine..... | 0.0 | 0.0 | 0.0 | 0.0 | .. | 25.3 | 36.0 | 0.6 | ... | 0.0 | 0.9 | 3.8 | 0.4 | 0.5 |
| Alanine..... | 8.4 | 2.5 | 1.5 | 0.8 | 4.2 | 8.7 | 21.0 | 4.4 | ... | 2.0 | 4.7 | 3.6 | 2.1 | 1.4 |
| Valine..... | .. | 0.9 | 7.2 | 1.9 | .. | 0.0 | 0.0 | 2.8 | 4.3 | 3.4 | 0.2 | .. | .. | 0.2 |
| Leucine and isoleucine.... | 15.2 | 19.4 | 9.4 | 9.9 | 29.0 | 7.1 | 1.5 | 11.5 | ... | 6.6 | 6.0 | 20.9 | 8.0 | 4.5 |
| Aspartic acid..... | 6.1 | 1.0 | 4.1 | 2.2 | 4.4 | 3.4 | .. | 2.3 | ... | 0.6 | 0.9 | 4.5 | 5.3 | 5.4 |
| Glutamic acid..... | 14.0 | 10.1 | 21.6 | 13.0 | 1.7 | 5.8 | 0.0 | 12.9 | ... | 43.7 | 23.4 | 6.3 | 17.0 | 23.2 |
| Serine..... | .. | .. | 0.5 | .. | 0.6 | 0.4 | 1.6 | 0.1 | 7.8 | 0.2 | 0.7 | 0.3 | 0.5 | .. |
| Hydroxyglutamic acid.... | 1.4 | .. | 10.5 | .. | .. | 0.0 | .. | .. | .. | .. | .. | .. | .. | .. |
| Phenylalanine..... | 5.2 | 2.4 | 3.2 | 2.6 | 4.2 | 1.4 | 1.5 | 0 | ... | 2.4 | 2.0 | 2.4 | 3.8 | 2.5 |
| Tyrosine..... | 3.2 | 0.9 | 4.5 | 3.4 | 1.3 | 0.0 | 10.5 | 2.9 | ... | 1.2 | 4.3 | 2.1 | 1.6 | 1.1 |
| Tryptophan..... | .. | .. | 1.7 | .. | .. | .. | .. | .. | ... | 1.0 | .. | .. | .. | .. |
| Cystine..... | 0.4 | .. | .. | .. | 0.3 | .. | .. | 7.3 | ... | 0.5 | 0.02 | .. | .. | .. |
| Methionine..... | 2.5 | .. | 1.4 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. |
| Lysine..... | 5.0 | 9.2 | 6.0 | 5.4 | 8.1 | 5.9 | 0.3 | 2.3 | 0 | 0.2 | 1.9 | 2.2 | 5.0 | 0.7 |
| Arginine..... | 5.4 | 3.2 | 3.8 | 7.9 | 3.3 | 8.2 | 0.7 | 7.8 | 87.4 | 3.2 | 4.7 | 15.8 | 11.7 | 11.9 |
| Histidine..... | 1.4 | 2.1 | 2.5 | 1.2 | 7.6 | 0.9 | 0.1 | 0.7 | 0.0 | 0.6 | 1.8 | 2.1 | 2.4 | 1.6 |
| Proline..... | 4.2 | 4.0 | 8.0 | 4.2 | 2.3 | 9.5 | 0.3 | 4.4 | 11.0 | 13.2 | 4.2 | 1.7 | 3.2 | 2.5 |
| Hydroxyproline..... | .. | .. | 0.3 | .. | 1.0 | 14.1 | .. | .. | ... | .. | .. | 2.0 | .. | .. |
| Ammonia..... | .. | 1.3 | 1.6 | 1.3 | .. | 0.4 | .. | .. | ... | 5.2 | 4.0 | .. | 2.1 | 3.7 |
| Total..... | 72.4 | 57.0 | 87.8 | 53.8 | 68.0 | 91.3 | 73.5 | 60.0 | 110.5 | 84.0 | 59.72 | 61.4 | 63.1 | 59.2 |

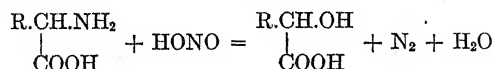
The Results of Hydrolysis.—Except for the figures for the diamino acids, the data cannot be regarded as quantitative. Reference to the quantities of tyrosine, cystine and tryptophan obtained by colorimetric estimation shows that the figures by isolation are considerably smaller. The low yields arise from the methods of isolation and from the use of the ester method for separating the monoamino acids which entails unavoidable losses. A new method has been described for isolating proline and hydroxyproline by means of ammonium reineckate, but the yields are not greater than by the butyl alcohol or ester method.

Several striking peculiarities in the composition of proteins are noticeable. Salmin and other proteins of this group (the protamins) are composed mainly of diamino acids. The gliadins of cereals, such as the gliadin of wheat, contain much glutamic acid and they have a high proportion of proline and very little of the diamino acids. The glutenins of cereals are also low in diamino acids and contrast with

the globulins of hemp seed, pea and almond, which have a high amount of arginine. These vegetable proteins have also very little cystine. The animal proteins generally contain all the amino acids in more even amounts. In consequence, they are often called "complete" proteins and are of better value in nutrition than the vegetable proteins which may be regarded as "disproportionate" proteins. Hemoglobin contains much histidine. Gelatin lacks tyrosine, cystine and tryptophan and is an "incomplete" protein. It has a high amount of glycine. Silk fibroin has more glycine, alanine and tyrosine than any protein so far examined. The keratins have the highest amounts of cystine.

A study of the diamino acid content of the keratins by Vickery and Block⁷⁵ has revealed that these proteins contain histidine, lysine and arginine in the molecular ratios of 1:4:12. With the modern work on the molecular weight of proteins which shows that they possess a numerical size of about 34,500, or a multiple, the number of amino acid units in such a molecule is about 250; and it is possible to calculate the number of those units which can be estimated with fair accuracy. Thus, Vickery and Leavenworth (1928) give 33 molecules of histidine, 13 molecules of arginine and 37 molecules of lysine in hemoglobin, with a molecular weight of 66,800. Calvery¹³ has calculated 2 molecules of tryptophan, 2 molecules of cystine, 10 molecules of arginine, 14 molecules of lysine, 5 molecules of histidine and 12 molecules of proline in a molecule of crystalline ovalbumin of a molecular weight of 34,000.

Analysis by Distribution of Nitrogen.—The amino acids which arise from proteins by hydrolysis belong to several different groups. Two main groups are distinguished: The basic or diamino acids and the monoamino acids. The former group can be precipitated by phosphotungstic acid together with cystine. The latter group, with which proline and hydroxyproline are associated, is not precipitated. The compounds in both groups can be further differentiated by their behavior with nitrous acid. Those amino acids with an amino group yield nitrogen according to the equation:



Proline and hydroxyproline do not react and are estimated together in the monoamino fraction. All the diamino acids react with nitrous acid, but they can be differentiated by other reactions. Arginine is decomposed by boiling with alkali with liberation of ammonia; cystine is differentiated by its sulfur content. Besides the amino acids, ammonia is liberated from amide groups, and owing to decomposition of tryptophan a certain amount of humin is formed. This subdivision of amino acids was first pointed out by van Slyke⁷³ who devised a simple procedure and a convenient apparatus for determining amino nitrogen. The details must be referred to in the original papers

of Van Slyke. They were carefully examined by Plimmer, Rosedale and Lowndes⁶² who improved the method of making the sulfur estimation.

The following estimations are made:

1. Amide nitrogen by estimating the ammonia after evaporating the solution and making alkaline with excess of slaked lime.

2. Humin nitrogen precipitated by the excess of slaked lime which is filtered off.

3. The phosphotungstic acid precipitate is filtered off and dissolved in alkali:

(a) Total nitrogen in a portion of the solution.

(b) Arginine nitrogen. A portion of the solution is boiled with sodium hydroxide and the ammonia evolved estimated. The arginine nitrogen is twice the amount of nitrogen evolved on boiling with alkali.

(c) Amino nitrogen with nitrous acid in another portion. Arginine yields one quarter of its nitrogen; histidine yields one third of its nitrogen. Lysine and cystine yield the whole of their nitrogen.

DISTRIBUTION OF NITROGEN IN PROTEINS

| | <i>Amide</i> | <i>Humin</i> | <i>Cys- tine</i> | <i>Argi- nine</i> | <i>Histi- dine</i> | <i>Ly- sine</i> | <i>Mono- amino</i> | <i>Mono- non- amino</i> | <i>Total</i> |
|------------------|--------------|--------------|----------------------|-----------------------|------------------------|---------------------|------------------------|---------------------------------|--------------|
| Casein..... | 10.27 | 1.28 | 0.2 | 7.41 | 6.21 | 10.3 | 55.81 | 7.13 | 98.61 |
| Egg yolk..... | 9.0 | 2.4 | ... | 14.5 | 3.1 | 9.4 | 60.6 | 1.6 | 100.6 |
| Egg white..... | 9.2 | 2.0 | ... | 11.7 | 0.2 | 10.1 | 66.0 | 1.6 | 100.9 |
| Hemoglobin..... | 5.24 | 3.6 | 0 | 7.7 | 12.7 | 10.9 | 57.0 | 2.9 | 100.04 |
| Gelatin..... | 2.25 | 0.07 | 0 | 14.7 | 4.48 | 6.32 | 55.8 | 14.9 | 99.02 |
| Lactalbumin..... | 7.72 | 1.65 | 2.34 | 7.27 | 4.2 | 13.08 | 60.91 | 2.03 | 99.2 |
| Edestin..... | 9.99 | 1.98 | 1.49 | 27.05 | 5.75 | 3.86 | 47.55 | 1.7 | 99.37 |
| Gliadin..... | 25.52 | 0.86 | 1.25 | 5.71 | 5.2 | 0.75 | 51.98 | 8.5 | 99.77 |

The nonamino nitrogen of these four compounds belongs to the arginine and histidine. With the amount of arginine known from the decomposition by alkali, the histidine nitrogen can be calculated.

(d) Sulfur estimation of a portion of the solution. From this value the cystine nitrogen is ascertained.

4. The filtrate from the phosphotungstic acid precipitate:

(a) Total nitrogen in a portion of the solution.

(b) Amino nitrogen in another portion of the solution.

Proteins from all sources have been analyzed by this method on account of its simplicity. They must be isolated so as to be free from other nitrogenous compounds. Hydrolysis in presence of carbohydrate gives too high results for humin nitrogen owing to decomposition of amino acids under these conditions. The proteins in foodstuffs cannot be analyzed in this way.

The values for the diamino acids are generally higher than the values given by the Kossel-Patten method or by the Vickery-Leavenworth method, and are usually considered more accurate. The conversion of the nitrogen values to amounts of amino acids can be calculated from the formulae and from the amount of nitrogen in the protein. The distribution of nitrogen in several proteins is given in the table on page 160.

The Estimation of Tyrosine and Tryptophan.—Folin and Denis³⁸ worked out a method of estimating tyrosine depending on the blue color which it gives with a phenol reagent consisting of phosphotungstic-

THE AMOUNTS OF TYROSINE AND TRYPTOPHAN IN PROTEINS

| Protein. | | Folin and Looney | Looney | Folin and Ciocalteu | Folin and Marenzi | May and Rose* | Jones, Gersdorff and Moeller |
|--------------------------|------------|------------------|--------|---------------------|-------------------|---------------|------------------------------|
| Casein..... | Tyrosine | 5.32 | | 6.46 | | 2.3 | 2.2 |
| | Tryptophan | 1.54 | | 1.4 | | | |
| Ovalbumin.... | Tyrosine | 4.2 | 4.1 | 4.0 | 3.98 | 1.6 | |
| | Tryptophan | 1.23 | 1.26 | 1.33 | 1.18 | | |
| Gelatin..... | Tyrosine | 0 | 0 | 0 | 0 | | |
| | Tryptophan | 0 | 0 | 0 | 0 | | |
| Edestin..... | Tyrosine | 5.7 | 4.58 | 4.53 | 4.54 | 2.2 | 2.48 |
| | Tryptophan | 1.4 | 1.52 | 1.51 | 1.46 | | |
| Gliadin..... | Tyrosine | 3.4 | 3.04 | 3.1 | 3.39 | 1.5 | 1.01 |
| | Tryptophan | 1.14 | 1.1 | 0.84 | 0.84 | | |
| Glutenin..... | Tyrosine | 4.5 | 4.56 | | | 2.6 | 1.72 |
| | Tryptophan | 1.68 | 1.64 | | | | |
| Zein..... | Tyrosine | 5.52 | 5.66 | 5.9 | 5.88 | 0 | 0 |
| | Tryptophan | 0 | 0 | 0.17 | 0.2 | | |
| Cryst. serum albumin.... | Tyrosine | | | | 4.66 | | |
| | Tryptophan | | | | 0.53 | | |
| Cotton seed globulin.... | Tyrosine | | | | 3.60 | | |
| | Tryptophan | | | | 1.39 | | |
| Muscle globulin | Tyrosine | | | | 3.92 | | |
| | Tryptophan | | | | 0.98 | | |
| Hemoglobin.... | Tyrosine | | | | 3.15 | | |
| | Tryptophan | | | | 1.28 | | |
| Serum globulin. | Tyrosine | 6.7 | | | | | |
| | Tryptophan | 2.28 | | | | | |
| Fibrin..... | Tyrosine | 6.5 | | | | | |
| | Tryptophan | 2.9 | | | | | |
| Wool..... | Tyrosine | 5.52 | | | | | 4.4 |
| | Tryptophan | 1.45 | | | | | |
| Horn..... | Tyrosine | 5.28 | | | | | |
| | Tryptophan | 1.43 | | | | | |
| Ovovitellin.... | Tyrosine | | | | | 2.6 | 2.42 |
| | Tryptophan | | | | | | |

* These are May and Rose's figures multiplied by 1.47 to correspond with 2.2 per cent tryptophan in casein.

phosphomolybdic acid. The results were later found to be too high owing to the decomposition products of tryptophan. Folin and Looney⁴⁰ altered the method and at the same time estimated trypto-

phan, which also gives a blue color with the phenol reagent but 0.887 times as intense. For the purpose of estimating both compounds, the protein is hydrolyzed with alkali and the tryptophan removed by precipitation with mercuric sulfate in sulfuric acid solution. The estimation of tyrosine is made on the filtrate and that of tryptophan on the precipitate which is dissolved in hydrochloric acid. There was much criticism of the method, to which Looney⁵⁷ replied; at the same time he gave some amended data. Folin and Ciocalteu³⁹ found that the tyrosine could be accurately estimated by Millon's reaction and published further data on both tyrosine and tryptophan. Folin and Marenzi⁴¹ adapted the method for determinations in 0.1 Gm. of protein and described a better reagent for estimating tryptophan. The values found by these workers are given in the table on page 161.

The color reaction which tryptophan gives with *p*-dimethylaminobenzaldehyde was adapted by May and Rose⁵⁸ for its estimation. The blue color so obtained was matched against the blue color given by casein treated in the same way as the protein under examination. The tryptophan content of casein was taken as 1.5 per cent as standard. Jones, Gersdorff and Moeller⁴⁸ used this method for determining the tryptophan content of a large number of proteins but took the value of 2.2 per cent as the amount in casein, a value given by other colorimetric methods and probably more correct, since 1.7 per cent of tryptophan has been isolated from casein. The values are given in the table for comparison with the figures obtained by Folin and his coworkers.

The figures for tyrosine, except the earlier ones for casein and edestin by Folin and Looney, are very consistent and probably represent the real values in proteins. The figures for tryptophan by the method of May and Rose are generally higher than those by the Folin and Looney method, and seem definitely too high. The accuracy of the data by Folin and his collaborators is confirmed from determinations of molecular weights which agree with those deduced by physico-chemical methods by Cohn, Hendry and Prentiss.¹⁵

The Estimation of Cystine.—Folin and Looney⁴⁰ published a method for the estimation of cystine based on the color which cystine gives with the uric acid reagent of Folin and Denis. This method was improved by Folin and Marenzi,⁴¹ who eliminated the influence of tyrosine upon the reaction by using a modified phosphotungstic acid reagent. Sullivan⁶⁸ described a distinctive test for cysteine and later with Hess⁷⁰ used it for estimating cystine in a number of proteins. It depends upon the formation of cysteine with 1,2-naphthoquinone-4-sodium sulfonate of a bright red color, which is not discharged by reducing agents.

Another method of estimating cystine is to reduce it with zinc to cysteine, which is then titrated in acid solution with iodine (Okuda). This method also estimates cysteine in combination in glutathione, and SH or S—S groups in other compounds. The following table shows the values for cystine obtained by the different methods:

THE AMOUNTS OF CYSTINE IN PROTEINS

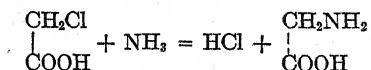
| | Folin and Looney | Folin and Marenzi | Okuda | Sullivan and Hess |
|---------------------------|------------------|-------------------|-------|-------------------|
| Casein..... | 0.25 | 0.3 | 0.31 | 0.31 |
| Gliadin..... | 2.32 | 2.19 | 2.04 | 2.07 |
| Edestin..... | 0.84 | 1.36 | 1.17 | 1.07 |
| Zein..... | 0.75 | 1.03 | | |
| Cryst. egg albumin..... | 0.81 | 1.23 | | |
| Cryst. serum albumin..... | | 6.06 | | |
| Fibrin..... | 3.5 | | | |
| Gelatin..... | 0.17 | | | |
| Zein..... | 0.75 | | | |
| Horn..... | 6.67 | | | |
| Wool..... | | 14.04 | 12.77 | 12.82 |

Synthesis and Constitution of the Amino Acids.—All the amino acids which are considered to be definite components of the protein molecule have been synthesized to prove their constitution. Except in the cases of alanine and proline, the synthesis has been effected after the isolation of the amino acid. The discovery of a new amino acid has been a stimulus to its synthesis and has led to the evolution of new methods. The synthesis of the amino acids is an important part of the chemistry of the proteins.

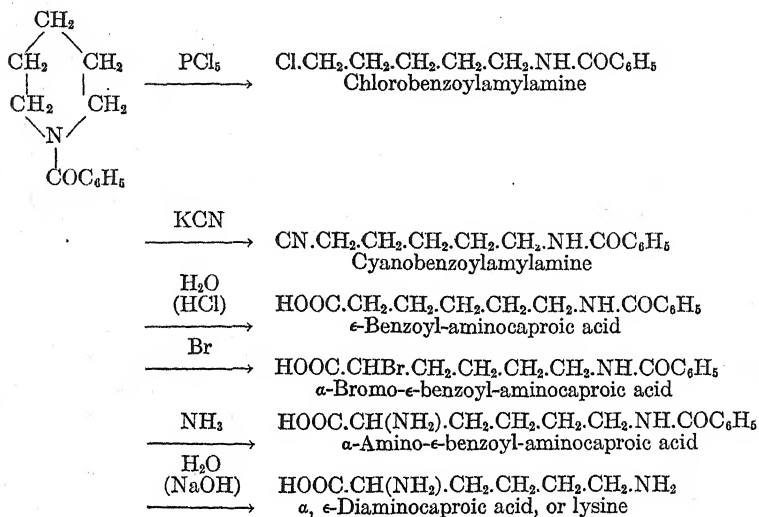
There are four general methods of synthesis of the amino acids with modifications necessitated by difficulties arising from inaccessibility of starting material or poor yields. The general methods are:

(1) The action of ammonia upon the corresponding halogen-substituted fatty acid; (2) the addition of hydrogen cyanide and ammonia to the lower aldehyde, followed by the hydrolysis of the nitrile group; (3) the synthesis by means of hippuric acid and reduction of the unsaturated condensation compound; and (4) the reduction of the corresponding nitroso, or imino, compound.

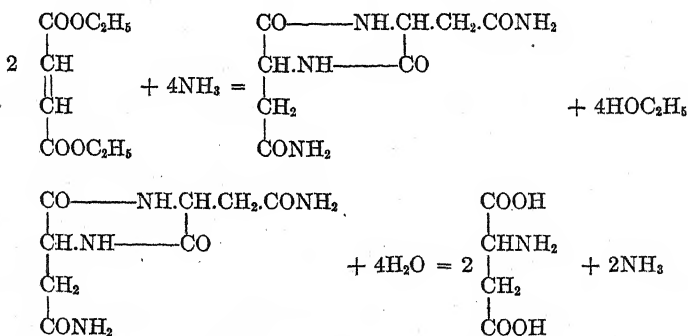
Method 1.—This method has been used for synthesizing glycine from chloracetic acid; alanine from α -chloro- or α -bromopropionic acid; valine from α -bromo-isovaleric acid; leucine from α -bromo-isocaproic acid; norleucine from α -bromocaproic acid; lysine from α -bromo- ϵ -benzoylamino-caproic acid; aspartic acid from ethyl fumarate is of value for preparing glycine:



Lysine is best made by this method starting from benzoylpiperidine:^{12a}

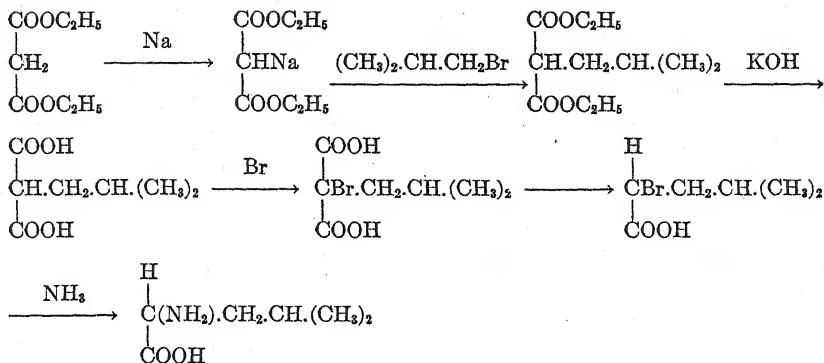


The synthesis of aspartic acid from ethyl fumarate²¹ may be included under this method. In this reaction the diamide of the diketo-piperazine of aspartic acid is formed as shown by Fischer and Koenigs³⁶ and is easily hydrolyzed to aspartic acid:

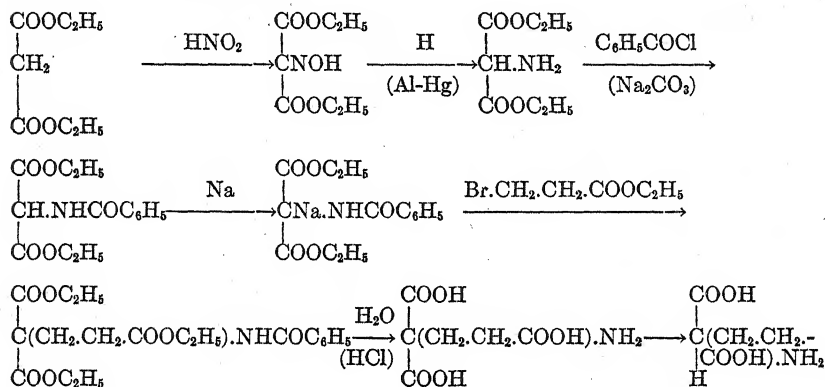


Indirectly, by the use of ethyl malonate, this method is the most convenient for preparing leucine from isobutyl bromide; isoleucine from sec. butyl iodide; phenylalanine from benzyl bromide; proline from trimethylene dibromide; hydroxyproline from epichlorhydrin; histidine from chloromethyl-glyoxaline; glutamic acid from ethyl bromopropionate; methionine from β -methylthioethyl chloride.

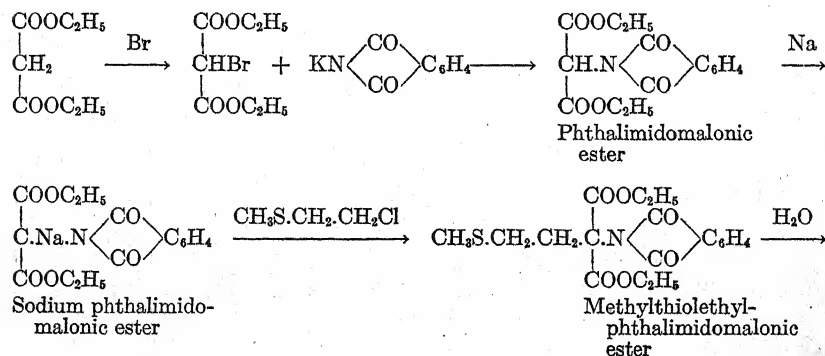
The several steps in the synthesis may be illustrated by the synthesis of leucine by Fischer and Schmitz:

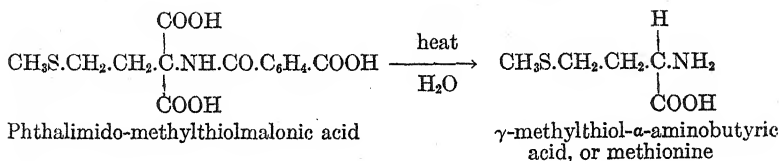


Dunn, Smart, Redemann and Brown²³ have prepared glutamic acid by this method as follows:

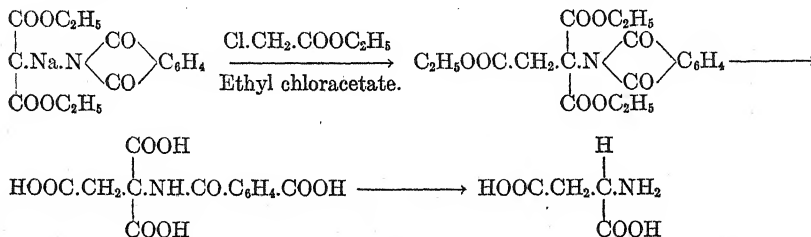


A valuable modification involving the introduction of potassium phthalimide was introduced by Sørensen⁶⁷ for the synthesis of phenylalanine, proline and other amino acids. It was used by Barger and Weichselbaum⁵ for the synthesis of methionine from α -methylthiolchloroethane:



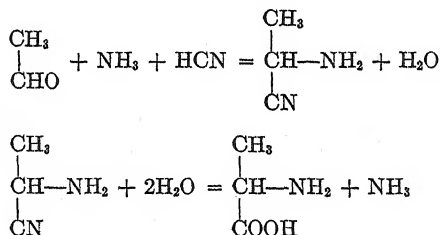


Dunn and Smart²² made aspartic acid by this method:



Method 2.—Strecker introduced this method in 1850 and synthesized alanine. It has been used for glycine from formaldehyde; alanine from acetaldehyde; valine from isobutyraldehyde; leucine from isovaleraldehyde; isoleucine from methyl-ethyl-acetaldehyde; serine from glycolic aldehyde and from chloroacetal; phenylalanine from phenylacetaldehyde; and methionine from β -methylthiolpropaldehyde.

The preparation of alanine is usually effected by this method:

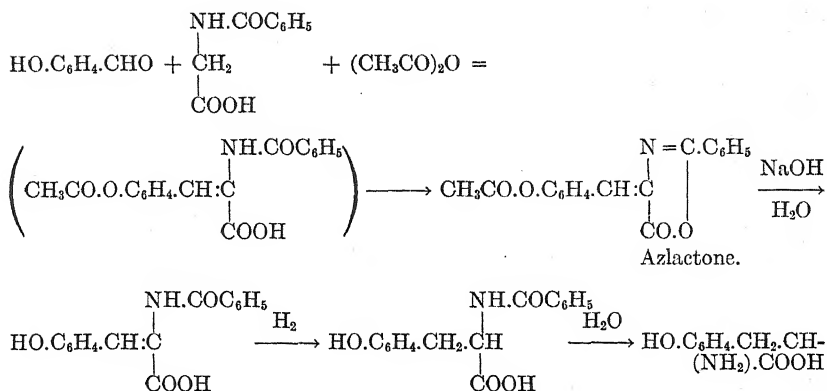


The yields are good. They are improved by following the directions of Cocker and Lapworth.¹⁴

Anslow and King⁴ showed that the first reaction in the case of glycine gives methylene amino-acetonitrile $(\text{CH}_2:\text{N}\cdot\text{CH}_2\cdot\text{CN})_3$, which is converted by alcoholic sulfuric acid into amino-acetonitrile. It separates out as its hydrogen sulfate and on boiling with baryta yields glycine.

Method 3.—This method with hippuric acid was introduced by Erlenmeyer in 1882, and is the best for synthesizing phenylalanine from benzaldehyde; tyrosine from *p*-hydroxybenzaldehyde; tryptophan from β -indolylaldehyde; serine from formic ester and sodium ethylate; and leucine from α -benzoylamino- β -isopropylacrylic acid.

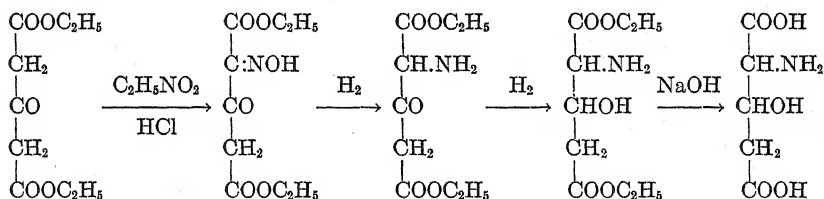
The synthesis of tyrosine proceeds in the following stages, the hydroxyl group being acetylated in the first stage and the acyl group removed on hydrolysis of the azlactone:



The synthesis of tryptophan has been simplified and improved by Lamb and Robson.⁵⁵

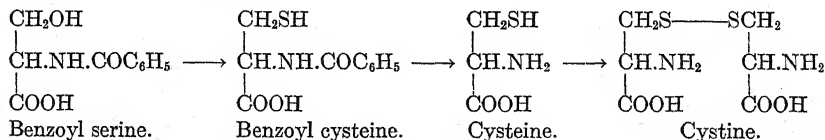
Method 4.—Several of the amino acids have been synthesized by the reduction of the oximino compound; for example, leucine from α -oximino-isobutylacetic acid; isoleucine from sec.-butyl-acetoacetic ester; aspartic acid from sodium oxalacetic ester; glutamic acid from α -nitroso-glutaric acid; and hydroxyglutamic acid from isonitroso-acetone dicarboxylic ester.

Hydroxyglutamic acid has been prepared in good yield by Harington and Randall⁴⁴ as follows:



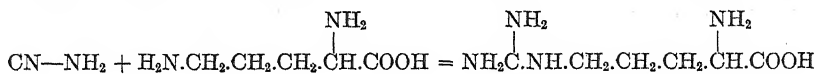
Special methods are necessary for the syntheses of cystine, arginine, 3, 5-diiodotyrosine and thyroxine.

Cystine.—Cystine, the reduction product of cystine, is prepared by the action of barium hydrosulfide, or phosphorus pentasulfide, on benzoylserine. By mild oxidizing agents, or air, cystine is easily converted into cystine:



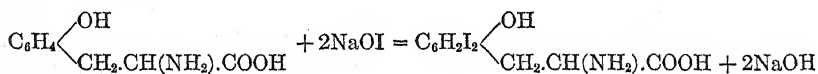
Arginine.—Like all compounds derived from guanidine, arginine is prepared by the action of cyanamide upon the corresponding amine, ornithine. Ornithine is prepared by the malonic ester synthesis using

the Sørensen variation. It is more easily prepared from benzoylpyridine, which is oxidized by permanganate to benzoyl- δ -aminovaleric acid. Ornithine is then made by the same reactions which yield lysine.

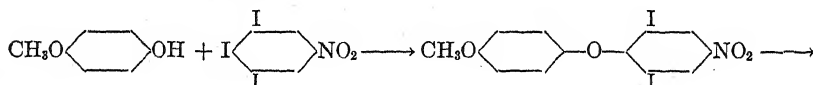


Sørensen proved the correctness of this reaction by the synthesis with cyanamide and α -monobenzoylornithine.

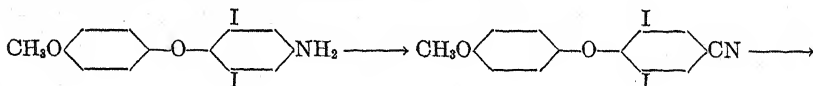
3, 5-Diiodotyrosine, or Iodogorgoic Acid.—This compound was synthesized by Wheeler and Jamieson⁸¹ by the action of the calculated quantity of iodine in two molecular proportions of sodium hydroxide upon *l*-tyrosine:



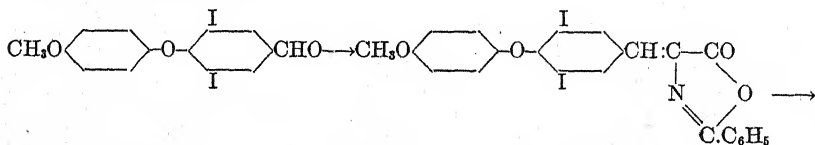
Thyroxine.—Harington and Barger⁴³ synthesized thyroxine by the following series of reactions: Quinol monomethyl ether was condensed with 3, 4, 5-triiodonitrobenzene to yield 3, 5-diiodo-4-(4'-methoxyphenoxy)-nitrobenzene:



This compound was reduced to the aniline by means of stannous chloride in hydrochloric acid. The diazonium salt was produced by amyl nitrite in dry hydrochloric acid and changed to the benzonitrile by copper sulphate and potassium cyanide:



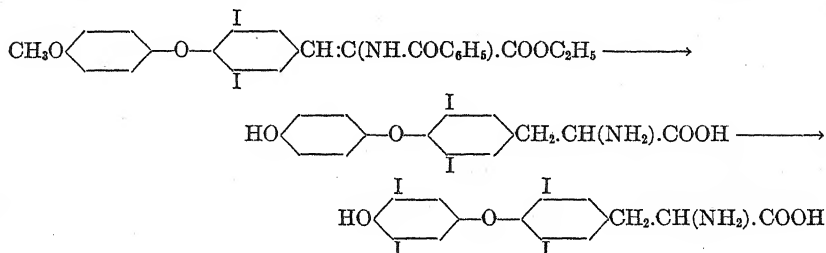
By treatment of the nitrile with anhydrous stannous chloride the aldehyde was obtained, from which, by the hippuric acid reaction, the azlactone was prepared.



On hydrolysis with alcohol containing 1 per cent sulfuric acid α -benzoylamino-3,5-di-iodo-4-(4'-methoxyphenoxy)-cinnamic ester was obtained.

Reduction with hydriodic acid and red phosphorus, and hydrolysis, then gave β -(3,5-diiodo-4-(4'-hydroxyphenoxy)-phenyl)- α -amino-pro-

pionic acid into which the remaining two iodine atoms were introduced by slowly adding iodine dissolved in conc. ammonium hydroxide:



Though the syntheses of the amino acids generally proceed through many stages, yet it is easier to make many of them by synthesis than by isolation from the mixture of products resulting on hydrolysis. The following lists show the best methods of preparing the amino acids:

By synthesis

Glycine
Alanine
Valine
Leucine
Isoleucine
Norleucine
Serine
Phenylalanine
Aspartic acid
Hydroxyglutamic acid
Methionine

By isolation from proteins

Tyrosine
Cystine
Tryptophan
Histidine
Lysine
Arginine
Glutamic acid
Proline
Hydroxyproline
Methionine

Separation of Racemic Amino Acids.—Except glycine, all the amino acids contained in proteins are in the optically active form. The inactive, or racemic, form obtained by synthesis requires separation into the optically active forms in order that the real synthesis of the natural amino acid be accomplished.

The method of direct crystallization and mechanical removal of the two isomers has been effected in the case of the asparagines, from which *d*- and *l*- aspartic acids are obtained by hydrolysis. The separation of the optical isomers by means of micro-organisms or yeasts has been effected in the cases of *l*-glutamic acid, *d*-aspartic acid, *d*-leucine, *d*-cystine, *l*-alanine, *l*-valine. These compounds are, however, the optical antipodes of the natural amino acids. The direct crystallization of the amino acids with optically active bases is not possible on account of the small affinity of the amino acids with the bases, even in the cases of the dibasic acids.

By increasing the acidic character of the amino acids by preparing the benzoyl derivatives, Fischer found that stable salts were formed with various alkaloids. These salts were soluble with greater difficulty and were easily isolated. The isomers of alanine, leucine, phenylalanine, tyrosine, aspartic acid and glutamic acid were separated in this way.

The benzoyl derivative is prepared by shaking the amino acid in presence of sodium carbonate with excess of benzoyl chloride. The benzoyl group is subsequently easily removed by hydrolysis with acid.

Fischer subsequently found that the formyl derivative was more useful than the benzoyl, and separated phenylalanine, leucine and valine in this way. The formyl derivative is prepared by heating the amino acid with anhydrous formic acid, and it is very easily hydrolyzed back to the amino acid. The formyl compound was used by Windus and Marvel for separating the isomers of methionine.

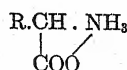
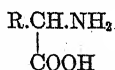
In the cases of proline and serine, the nitrobenzoyl compound was used for the separation of the isomers.

Except for histidine, the diamino acids have not yet been separated into their optical isomers. Pyman separated histidine by means of the tartrates.

Cystine, hydroxyproline and hydroxyglutamic acid contain two asymmetric carbon atoms. The separation of the four stereo-isomers has not yet been accomplished.

Walden Inversion.—A change in rotation arises in the conversion of the halogen substituted fatty acid into the corresponding amino acid; *e. g.*, *d*-bromopropionic acid yields *l*-alanine, and *l*-valine is obtained from *d*-bromovaleric acid. This change in rotation, or Walden inversion, is important in the correlation of the configurations of the amino acids. A change of configuration occurs in several other series of organic compounds and is beyond the scope of this chapter. With reference to the rotations of the amino acids, it has been proved that though the actual rotations may be dextro- or levo-, the arrangement of the groups around the asymmetric carbon atom is the same. Thus, *d*-alanine, *l*-serine, and *l*-cystine have the same configuration.

The Properties of the Amino Acids.—The amino acids are colorless, crystalline compounds often with characteristic forms which may be used in identification, *e. g.*, in the cases of cystine and tyrosine. Except for tyrosine and cystine, the amino acids are easily soluble in water, and except for proline, they are not soluble in absolute alcohol. They generally dissolve in aqueous alcohol and acetone. They are not soluble in ether and other organic solvents. According to the number of amino or acid groups in the molecule, the amino acids are neutral, basic or acid in reaction. They form salts with metallic bases of which the copper salts of the monoamino acids and the silver salts of the diamino acids are the most characteristic. Salts are also formed with strong acids. Derivatives with acid chlorides and phenylisocyanate can be made which serve to identify the amino acids. The simple amino acids exist in solution in two forms:

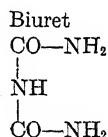
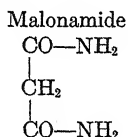
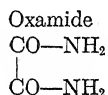


in the first of which they behave as amphoteric electrolytes and in the second as "zwitterions."

The Polypeptides, Diketopiperazines.—The researches of Emil Fischer and of Kossel upon the simple monoamino acids and the diamino acids showed quite definitely that the proteins were composed of numerous amino acids combined together in different proportions. The possible ways of combination of the amino acids were reviewed by Hofmeister in 1902. With the unlikelihood of combination through their carbon atoms, there remained the chief possibility of combination by means of the nitrogen atoms, and three ways were then possible: (a) $-\text{CH}_2-\text{NH}-\text{CH}_2-$; (b) $-\text{CH}_2-\text{NH}-\text{CO}-$; (c) $-\text{CH}_2-\text{NH}-\text{C}(=\text{NH})-$.

The linking of carbon atoms by an imino group as in (a) is present in proline and hydroxyproline. Its occurrence to any larger extent in the protein molecule is unlikely, as many free carboxyl groups would be left of which there is no evidence. Arginine contains the guanidine linking shown in (c) and there is no evidence of any more groupings of this basic nature in the proteins.

The acid amide mode of combination shown in (b) is in every way the most probable. It leaves a molecule without any large number of amino or carboxyl groupings, with a neutral character, and is easily broken by hydrolysis with the liberation of an equal number of acid and basic groups. The formation of ammonia on hydrolysis also suggests the acid amide grouping $-\text{CONH}_2$. This linking is found in simple compounds such as:



which give the biuret reaction. These compounds contain two $\text{CO}-\text{NH}$ groups joined directly, or by a carbon atom, or by a nitrogen atom.

Three amino acids joined together in this way, $\text{H}_2\text{N}.\text{CHR}.\text{CO}-\text{NH}.\text{CHR}.\text{CO}-\text{NH}.\text{CHR}.\text{COOH}$, have two $\text{CO}-\text{NH}$ groupings and explain the biuret reaction of proteins. The formation of glycocholic acid and of hippuric acid in the living body supports the acid amide form of combination in the protein molecule. This type of combination further lends itself to the presence of other structures which can be easily derived from it. Other ways of linking amino acids may be present in proteins, such as the ester grouping between the hydroxyl group of a hydroxy acid and the carboxyl group of any amino acid. The ether grouping is not excluded, nor is the anhydride grouping. These groupings would give a basic character to the protein. They are not likely to be present.

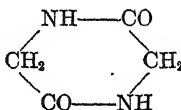
The Polypeptides or Peptides.—Anhydrides of various complexity can be made by heating together amino acids with or without dehydrating agents. This method of combination does not give any indication of their structure.

The systematic work of Emil Fischer begun in 1901 on the combination of 2, 3 or more amino acids showed clearly that the acid amide form of combination was the chief mode of linking of the amino acids. These combinations were termed the polypeptides, or more shortly the peptides. This word was derived from the substance peptone which is produced by the action of pepsin on proteins. By the introduction of the Greek numerals di-, tri-, tetra-, etc., it indicated how many amino acids were combined together in the synthetical substance.

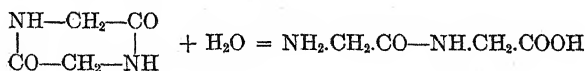
Fischer devised three methods for preparing polypeptides: (1) The formation of anhydrides, the diketopiperazines, and their hydrolysis; (2) the action of the acid chloride of the corresponding halogen compound upon an amino acid or its ester, followed by the exchange of the halogen atom for the amino group; (3) the action of the acid chloride of an amino acid upon another amino acid, or upon a peptide.

A fourth method of preparing dipeptides was introduced in 1932 by Bergmann and Zervas⁹ in which the acid chloride of the benzylcarbonato derivative of an amino acid is combined with another amino acid.

Method 1.—Curtius and Goebel observed that if glycine ester were allowed to stand with water for a few days, it was converted into an anhydride for which the formula of diketopiperazine was proposed by Curtius and Schulze:



Fischer found that this anhydride was best obtained by heating the ester of glycine in a sealed tube at 150–180° for some hours. Fischer and Fournneau showed that the anhydride was hydrolyzed (by boiling with concentrated hydrochloric acid) and converted into the salt of a new amino acid, from which they prepared the free acid by means of silver oxide. The reaction is represented by the equation:



The new compound was the first anhydride of two molecules of glycine and was called glycyglycine. By means of hydrochloric acid in alcohol it was converted into its ester. Both the free acid and its ester were easily changed into the diketopiperazine.

The esters of alanine and of leucine were converted in a similar way into the diketopiperazines, which were hydrolyzed into alanylalanine and leucyl-leucine. Several other dipeptides can be made by this method.

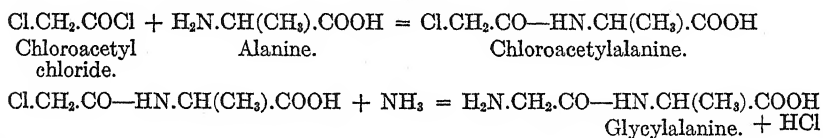
This method does not lend itself to the preparation of mixed anhydrides containing two different amino acids. The mixed anhydride on hydrolysis yields two different dipeptides which are very difficult to

separate; thus, alanylglycine and glycylalanine are obtained from glycyl-alanine anhydride.

The dipeptides have the constitution of an amino acid and can be converted into esters. These esters are, however, not suitable for preparing more complex anhydrides and the resulting more complex polypeptide.

The anhydride, or diketopiperazine, structure is believed to be present in the constitution of certain proteins. Several have been isolated from the products of hydrolysis of proteins.

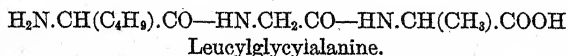
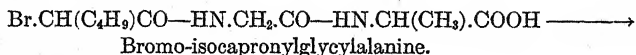
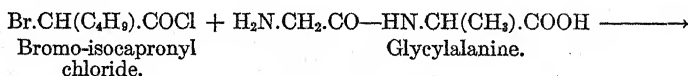
Method 2.—This method of synthesis of a peptide resembles the combination of an acid chloride such as benzoyl chloride with glycine. It consists in the use of a halogen substituted acid chloride which is combined with an amino acid or its ester in anhydrous solvents and the exchange of the halogen radical with ammonia. It was described by Fischer and Otto.³³ The reaction is:



The acid chlorides available for this synthesis are: α -Bromopropionyl chloride for the alanyl radical; α -bromo-isocaprolyl chloride for the leucyl radical; α -bromo-hydrocinnamyl chloride or phenyl-bromopropionyl chloride for the phenylalanyl radical; α , δ -dibromovaleryl chloride for the prolyl radical.

Some of these acyl chlorides can be procured in the optically active form. It is thus possible to synthesize dipeptides with the natural forms of the amino acids. In the synthesis, change of rotation, or Walden inversion, may take place during the reaction with ammonia so that the correct optically active form of the halogen compound must be used.

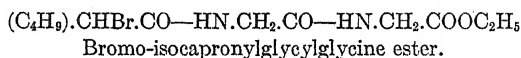
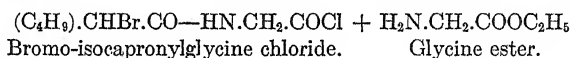
This reaction can be extended to the synthesis of a tripeptide, a tetrapeptide, a pentapeptide and other polypeptides, since the dipeptide, or tripeptide is an amino acid in constitution:



Leucyl-glycyl-alanine \rightarrow Alanyl-leucyl-glycyl-alanine \rightarrow Leucyl-alanyl-leucyl-glycyl-alanine \rightarrow Glycyl-leucyl-alanyl-leucyl-glycyl-alanine

Though numerous polypeptides have been made by this method, the procedure is limited in its application in two ways. The corresponding halogen acyl derivative for every amino acid is not available, and the chain of amino acids can be lengthened only at one end.

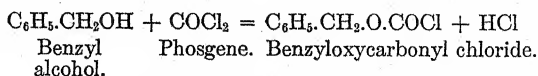
Method 3.—This method is the most direct for the synthesis of a dipeptide or polypeptide, and possesses the advantage that the chain of amino acids can be lengthened at either end of the peptide molecule, so that the amino acids can be united in every possible order. The chlorides of the amino acids were unknown at the beginning of Fischer's researches and all attempts to prepare them had failed. In 1904 it was found that the presence of a halogen acyl group in the molecule of an amino acid made the preparation of the acid chloride of this compound possible by the action of phosphorus pentachloride in the presence of acetyl chloride. Thus, bromo-isocapronylglycine chloride could be made and combined with the ester of an amino acid:

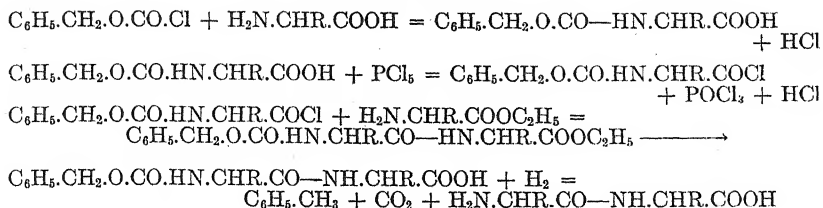


Removal of the ester group and treatment with ammonia then gave the tripeptide, leucylglycylglycine, $(\text{C}_4\text{H}_9)\text{CH}(\text{NH}_2)\cdot\text{CO}-\text{HN}\cdot\text{CH}_2\cdot\text{CO}-\text{HN}\cdot\text{CH}_2\cdot\text{COOH}$. If glycylglycine ester were used in combination with bromo-isocapronylglycine chloride, the tetrapeptide, leucyldiglycylglycine was obtained. By combining with esters of the peptides already made the synthesis of a heptapeptide, a decapeptide and finally of an octadecapeptide was effected. The latter compound contained 18 amino acid radicals of which three were leucine and it had a molecular weight of over 1200.

By the same method of preparation in acetyl chloride solution, the acid chlorides of many amino acids were subsequently made. Tyrosyl chloride was of value for introducing the tyrosine radical at the beginning of a chain. Every possible dipeptide and polypeptide can be made by this method, but their synthesis is not actually possible as the starting materials are difficult to procure and there are several practical difficulties in the process.

Method 4.—Bergmann and Zervas⁹ found that the acid chloride of an amino acid could be easily made if the amino group were protected by the introduction of the benzylcarbonato group. This acid chloride interacts with the ester of an amino acid giving the derivative of a dipeptide from which the benzylcarbonato group is removed by reduction. The stages in the synthesis are:





Dipeptides have been made from benzylcarbonatoglycyl chloride with alanine, serine, cystine, glutamic acid, phenylalanine, tyrosine, arginine, histidine. The extension of the method has given the rare and important dipeptides: *d*-glutamyl-*d*-glutamic acid; *l*-aspartyl-*l*-tyrosine; *d*-lysyl-*d*-glutamic acid; *d*-lysyl-*l*-histidine; *d*-alanyl-*l*-proline; glycyl-*l*-proline. Here we see a combination of two dibasic amino acids and two dicarboxylic amino acids and of the imino group of proline.

Properties of the Polypeptides.—The polypeptides are generally easily soluble in water, but those with phenylalanyl and leucyl radicals are not soluble. Except for leucyl-proline and other proline peptides, they are not soluble in absolute alcohol. They dissolve in dilute acids and alkalis, forming salts, and give blue copper salts with copper hydroxide. The tetrapeptides of glycine and other tripeptides give the biuret reaction. The higher peptides are precipitated by phosphotungstic acid, tannic acid and by concentrated solutions of ammonium sulfate. They are very much like natural proteins but differ in the absence of the special amino acids, tyrosine and tryptophan. They are in many cases hydrolyzed by the enzymes of the pancreas and of the small intestine.

Isomerism of the Peptides.—Various isomeric peptides can be prepared by synthesis. Two isomers exist in the case of the dipeptides; *e. g.*, alanylleucine and leucylalanine; and tyrosyl-leucine and leucyl-tyrosine.

Six isomers are possible in the case of tripeptides with the same three amino acids: Alanylglycylleucine; alanyl-leucylglycine; glycylalanylleucine; glycylleucylalanine; leucylglycylalanine; leucyl-alanylglycine. Twenty-four isomers are possible among the tetrapeptides such as tyrosylglycylalanylleucine; glycyltyrosylalanylleucine; alanyl-leucyltyrosylglycine; leucylalanyltyrosylglycine; etc. Not all of these have been made. As indicated above, the amino acid is not shown in its optically active form. If the amino acids are present in their racemic forms, the number of isomers is greater.

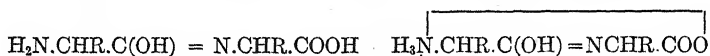
The synthesis of a natural protein is almost impossible on account of the immense number of isomers. There is no guide to the order in which they are combined. The isolation of some dipeptides and diketopiperazines from the products of mild hydrolysis of proteins indicates that these two are in combination. The action of enzymes on the synthetic peptides affords more help. The molecular weight

of the octadecapeptide is 1213. If this polypeptide contained tyrosine, phenylalanine and tryptophan units, its molecular weight would reach 3000 to 4000. According to the modern physical methods, the molecular weight of proteins is a multiple of 34,000, and the protein molecule would contain from 200 to 250 amino acids. The methods of synthesis have not yet been applied to the preparation of such complexes.

The Structure of the Polypeptides.—The structure of the polypeptides as shown by synthesis is that of substituted acid amides. Like the amino acids, these compounds may exist in two forms:



Both of these forms may also have the lactim arrangement:



The existence of isomeric forms of polypeptides was noticed during their synthesis. The first formula, which is the simplest, is commonly used as it is not possible to decide which is the exact structure.

The same type of isomerism will be shown by the diketopiperazines. These other types of diketopiperazines have been prepared by other methods and it is possible that some of the amino acids are present in diketopiperazine rings in the protein molecule.

The Action of Enzymes on the Polypeptides.—The action of trypsin and other proteolytic enzymes on many of the synthetic peptides was tested by Fischer and Abderhalden and Fischer and Bergell. Some were hydrolyzed, others were not hydrolyzed. Thus,

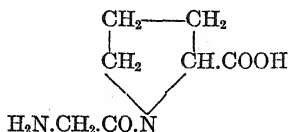
| <i>Hydrolyzed</i> | <i>Not hydrolyzed</i> |
|--------------------------------------|-------------------------------------|
| Alanylglycine | Glycylalanine |
| Alanyl-leucine | Leucylalanine |
| Glycyl-L-tyrosine | |
| Leucyl-L-tyrosine | |
| <i>d</i> -Alanyl- <i>d</i> -alanine | <i>d</i> -Alanyl- <i>L</i> -alanine |
| <i>d</i> -Alanyl- <i>L</i> -leucine | <i>L</i> -Alanyl- <i>d</i> -alanine |
| <i>d</i> -Alanyl- <i>L</i> -tyrosine | <i>L</i> -Leucyl-glycine |
| <i>L</i> -Leucyl- <i>L</i> -leucine | <i>L</i> -Leucyl- <i>d</i> -leucine |

These results show that only those dipeptides with the natural forms of the amino acids are hydrolyzed, and that the order of combination of the amino acids affects the action of the enzyme. Alanylglycine but not glycylalanine was hydrolyzed. There was, however, no regularity in the results.

The synthetic peptides have been of value to Willstätter and Waldschmidt-Leitz in dividing the proteolytic enzymes into several groups: Erepsin, the enzyme of the mucous membrane of the intestine, which hydrolyzes only dipeptides and some tripeptides; trypsin, which hydrolyzes the more complex peptone; trypsin kinase (formerly trypsin) which hydrolyzes the original protein and the peptone.

There is further subdivision into dipeptidase, aminopolypeptidase, carboxypolypeptidase.

The dipeptides prepared by Bergmann and Zervas were tested against enzymes. Dipeptidase hydrolyzed *d*-glutamyl-*d*-glutamic acid; *d*-lysyl-*d*-glutamic acid; *d*-lysylglycine; *d*-lysylhistidine; *d*-glutamyl-*l*-tyrosine but not *l*-aspartyl-*l*-tyrosine; glycyl-*l*-proline; *d*-alanyl-*l*-proline. The two latter were hydrolyzed by aminopolypeptidase. Examination of the hydrolysis of gelatin indicated that this form of combination of proline may be present in gelatin:

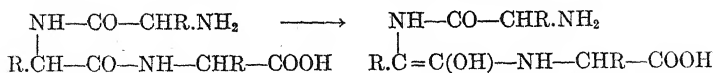


None of the polypeptides was hydrolyzed by pepsin. Fischer held the view that the polypeptide chain was not long enough for attack by pepsin, though other modes of combination of the amino acids might be present which could be hydrolyzed by this enzyme.

The Isolation of Peptides and Diketopiperazines from Proteins.—

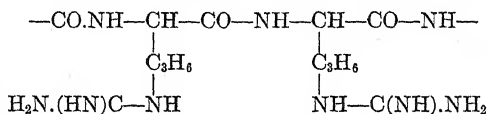
The order of combination of the amino acids in proteins is indicated to a certain extent by the isolation of some dipeptides and anhydrides from the products of mild hydrolysis. Many years ago Bopp isolated a compound which was termed leucinimide and identified as leucine anhydride. It would arise from leucyl-leucine. Fischer and Abderhalden³⁰ obtained from silk fibroin the diketopiperazines of glycine and alanine, and of glycine and tyrosine. The former was shown to be derived from glycylalanine and the latter from glycyltyrosine. The anhydride of glycine and leucine was isolated from edestin. A more complex anhydride consisting of two molecules of glycine, one molecule of alanine and one molecule of tyrosine was isolated from silk fibroin. From gliadin, *l*-leucyl-*d*-glutamic acid and *l*-prolyl-*l*-phenylalanine have been prepared, and from casein the anhydrides of *d*-alanine and *l*-phenylalanine and of *d*-alanine and *l*-leucine. It is probable that the amino acids are combined thus in the protein molecule. The combination in the anhydrides is not quite definite, as this type of compound is very easily produced by the reagents used in the isolation. All precautions were taken to avoid their secondary formation from amino acids and it is believed that certain anhydride rings exist in the proteins.

Hydrolysis of Proteins After Treatment with Alkali.—If proteins be dissolved in half normal alkali and the solution be kept, they undergo a change in rotatory power. The proteins suffer a mild hydrolysis but not to the stage of amino acids. On subsequent complete hydrolysis with acid, the amino acids are present partly in their optically active forms and partly in their racemic forms. Dakin considered that the alkali caused a keto-enol transformation:



while they were in combination. This transformation would not affect the carbon atom at the end of a chain. The amino acids at the end of a chain would be present in the mixture in the optically active forms. Several proteins have been treated in this way. Lysine, glutamic acid, part of the alanine and part of the proline were found in the optically active states, while leucine, aspartic acid, phenylalanine, arginine and histidine were isolated as racemic amino acids.

The Combination of the Basic Amino Acids.—Arginine, histidine and lysine can be combined with other amino acids either with their α -amino groups or with their special basic radical, or, as in the case of lysine, with the ϵ -amino group. Special investigations were made by Kossel to ascertain if the guanidine group of arginine was combined or free. Kossel and Kennaway nitrated clupein. Nitroclupein on hydrolysis yielded nitroarginine. It was therefore deduced that the guanidine grouping was free and not in combination. The structure of arginine in a molecule of clupein would thus be



Arginine in the molecules of other proteins is also not combined by its guanidine radical, but two arginine molecules are not necessarily combined with each other.

Proteins, on treatment with nitrous acid by the Van Slyke method, yield nitrogen. The amount so evolved is exactly half of that which is given by the lysine in the products of complete hydrolysis. It is concluded that only one of the two amino groups of lysine is present in acid amide combination with other amino acids, and that one amino group is free. This is confirmed by the reaction of proteins with formaldehyde which gives an amount corresponding to one free amino group of lysine, and by the fact that lysine cannot be isolated from proteins after they have been treated with nitrous acid. It is probable that the free amino group is the one in the ϵ -position.

From analogy it is considered that histidine is in combination with its amino group, and that its glyoxaline ring is free.

The Combination of the Dicarboxylic Amino Acids.—The three dicarboxylic acids, aspartic, glutamic and hydroxyglutamic can be combined by either or both of their carboxyl groups. Two isomeric forms of a dipeptide of a simple amino acid with one of these acids could exist.

Proteins on hydrolysis yield ammonia. Its origin is from simple acid amide groupings. A comparison of the amount of ammonia evolved on hydrolysis with the yields of the dicarboxylic acids shows

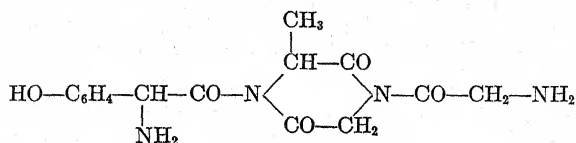
that there is a very close correspondence between the ammonia and one of the acid groups of these acids. Thus,

| | <i>Ammonia found</i> | <i>Ammonia calculated</i> |
|----------------|----------------------|---------------------------|
| Casein..... | 1.4 | 1.6 |
| Edestin..... | 2.2 | 2.3 |
| Phaseolin..... | 2.4 | 2.1 |
| Gliadin..... | 4.4 | 5.1 |
| Zein..... | 2.3 | 3.6 |
| Glutenin..... | 2.8 | 4.0 |

In some cases the amount calculated is greater than the ammonia found. Proteins are generally nearly neutral in character. This evidence would at any rate show that most of the acid groups were combined with ammonia. Physical methods support the presence of amide groupings with these acids. By analogy it is assumed that the carboxyl group next to the amino group is in peptide combination and that the other carboxyl group is free or combined with ammonia. This carboxyl group of glutamic acid is considered to be in combination in glutathione, and may be used to a certain extent in uniting amino acids in a polypeptide chain. The free carboxyl group may combine with hydroxyl groups of hydroxyamino acids, and it may be in salt formation with the basic groups of the basic amino acids. All amino acids are regarded as in combination with their α -amino groups. In the case of proline a part is in combination with its imino group and the carboxyl of another amino acid.

In the phosphoproteins the phosphoryl group appears to be combined with the hydroxyl group of a hydroxyamino acid. Products have been isolated indicating combination with serine and also with hydroxyglutamic acid.

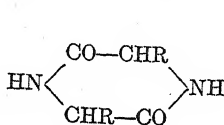
The Presence of Diketopiperazine Rings in Proteins.—The predominant form of union of the amino acids in proteins is undoubtedly that of the substituted amide which indicates that proteins consist of long chains of amino acids. Physical experiments on proteins demonstrate that proteins consist rather of rows of amino acids possibly held together by secondary valencies, and that the molecule is compact. The configuration of the amino acids points to their presence in the form of spirals which would be capable of intertwining and producing a compact mass. There may be at the ends of a chain or spiral the structure of a diketopiperazine. It was noted above that diketopiperazine rings had been isolated from proteins and the pressure of such rings in proteins is strongly advocated by Abderhalden. Besides the isolation of the anhydrides of glycine and alanine, and of glycine and tyrosine from silk fibroin, evidence has been produced for the presence of the complex compound with tyrosine, alanine, and two molecules of glycine which is believed to have the structure



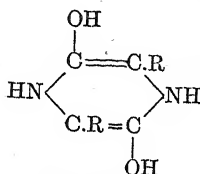
It closely resembles the tetrapeptide, glycyl-alanyl-glycyl-tyrosine. These products were identified by mild reduction with sodium and alcohol so as to stabilize the rings.

A series of color reactions for diketopiperazines was also worked out by Abderhalden and the fact that some of them yield oxamide on oxidation which can also be obtained by oxidation of proteins is additional evidence for their existence in proteins. It seems that they may be present in the proteins of the keratin class, but it is doubtful if they are in coagulable proteins.

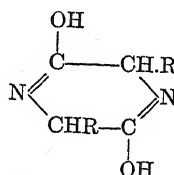
Isomeric Forms of the Diketopiperazines.—By the enolization of the keto groups, the diketopiperazines are capable of existence in two other modifications:



Ordinary keto form

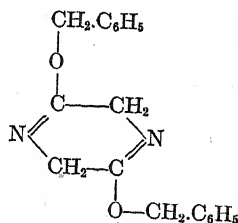


Enol form

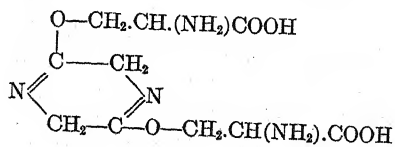


Pyrazine form

Substances of the pyrazine form have been prepared by Karrer and his colleagues. The benzyl derivative of glycine anhydride was made by the action of benzyl chloride on the silver salt of glycine anhydride:



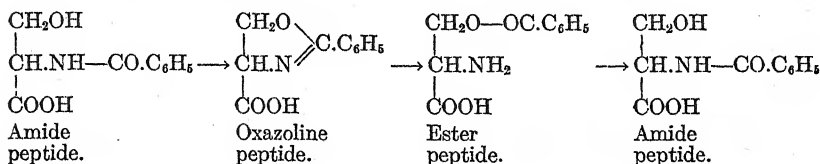
It was hydrolyzed by dilute acids into benzyl chloride and glycine. A compound in which alanine was joined to glycine anhydride



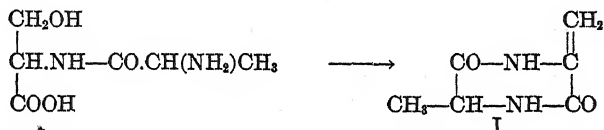
would give on hydrolysis glycine and serine. Serine might thus not actually be present in the protein molecule, though capable of being formed on hydrolysis.

There is no evidence for this type of combination but its possibility must not be overlooked. Other ring structures can be formed from the enol forms of dipeptides by ring closure.

Diketopiperazine Rings with Hydroxyamino Acids.—Bergmann and his pupils have studied dipeptides and diketopiperazines of serine, which show some remarkable and important transformations. Reference to benzoyl serine is first necessary. Benzoyl serine on treatment with thionyl chloride gives through loss of water and ring closure an oxazoline peptide. This peptide is stable in neutral or alkaline solution, but in acid solution the ring is opened and the isomeric benzoyl ester peptide is formed. Neutralization of the solution then gives the original

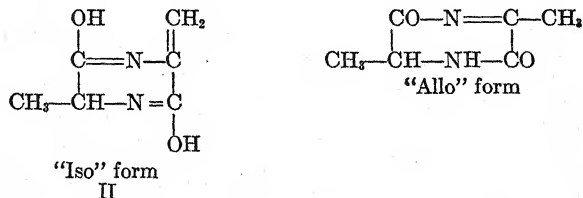


N-benzoyl serine. The changes with alanyl-serine and with glycyl-serine are more complicated. The dipeptide of alanyl-serine on treatment with thionyl chloride gives a base containing chlorine which forms an insoluble anhydride, I, of the formula $\text{C}_6\text{H}_8\text{O}_2\text{N}_2$. This anhydride, I, seemed to be 3-methylene-6-methyl-2, 5-diketopiperazine:



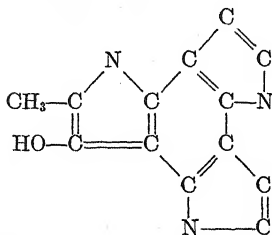
since it gave, on hydrolysis, pyruvylalanine, and, on reduction, alanine anhydride. It appears to have been formed from alanyl-serine by the loss of two molecules of water.

On solution in alkali and reprecipitation with acid, it was changed into another anhydride, II, which was shown to be isomeric with anhydride I. The formula



was given to the isomer. This "iso" form could pass into another "allo" form on heating with dilute ammonia and guanidine. The striking properties of lability and polymerization of these compounds may be an explanation of such properties of proteins as denaturation on heating and the insolubility of the keratins in the presence of enzymes. So far the presence of such groupings in proteins has not been indicated.

Pyrrole Rings.—The existence of pyrrole rings condensed together for the structure of proteins has been put forward by Troensegaard. The evidence for such rings is very scanty, but they would explain the formation of compounds of pyrrole on dry distillation of proteins and would connect together chlorophyll and hematin and proteins. It is possible to produce amino acids from such a structure, but not in accordance with the general mechanism of hydrolysis by enzymes which has been shown definitely to require acid amide linkings.



R. H. A. PLIMMER.

REFERENCES

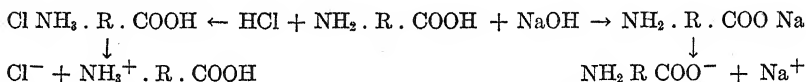
1. Abderhalden, E.: *Z. physiol. Chem.*, **128**, 119; **131**, 284; **154**, 18 (1923–1926).
2. Abderhalden, E., and Komm, E.: *Z. physiol. Chem.*, **134**, 121; **139**, 147, 181; **140**, 92, 99; **143**, 128 (1924–1925).
3. Abderhalden, E., and Schwab, E.: *Z. physiol. Chem.*, **139**, 169 (1924).
4. Anslow, W. K., and King, H.: *J. Chem. Soc.*, 2463 (1929).
5. Barger, G., and Weichselbaum, T. E.: *Biochem. J.*, **25**, 997 (1931).
6. Bergmann, M., and Köster, H.: *Z. physiol. Chem.*, **167**, 91 (1927).
7. Bergmann, M., Miekeley, A., and Kann, E.: *Z. physiol. Chem.*, **140**, 128 (1924); **146**, 247 (1925).
8. Bergmann, M., and Stather, F.: *Ann.*, **448**, 32 (1926).
9. Bergmann, M., and Zervas, L.: *Ber.*, **65**, 1192 (1932).
10. Bergmann, M., Zervas, L., and Greenstein, J. P.: *Ber.*, **65**, 1692 (1932).
11. Bergmann, M., Zervas, L., and Schleich, H.: *Ber.*, **65**, 1747 (1931).
12. Boyd, W. J.: *Biochem. J.*, **23**, 78 (1929).
- 12a. v. Braun, J.: *Ber.*, **42**, 839 (1909).
13. Calvery, H. O.: *J. Biol. Chem.*, **94**, 613 (1932).
14. Cocker, W., and Lapworth, A.: *J. Chem. Soc.*, 1391 (1931).
15. Cohn, E. J., Hendry, J. L., and Prentiss, A. M.: *J. Biol. Chem.*, **63**, 721 (1925).
16. Csonka, F. A., and Horn, M. J.: *J. Biol. Chem.*, **93**, 677 (1931).
17. Dakin, H. D.: *J. Biol. Chem.*, **13**, 357 (1912).
18. Dakin, H. D.: *Biochem. J.*, **12**, 290 (1918); **13**, 398 (1919).
19. Dakin, H. D.: *J. Biol. Chem.*, **44**, 499 (1920); **61**, 137 (1924).
20. Drechsel, E.: *J. prakt. Chem.*, **39**, 425 (1889).
21. Dunn, M. S., and Fox, S. W.: *J. Biol. Chem.*, **101**, 493 (1933).
22. Dunn, M. S., and Smart, B. W.: *J. Biol. Chem.*, **89**, 41 (1930).
23. Dunn, M. S., Smart, B. W., Redemann, C. E., and Brown, K. E.: *J. Biol. Chem.*, **94**, 599 (1931).
24. Erlenmeyer, E., and Halsey, J. T.: *Ann.*, **307**, 138 (1899).
25. Fischer, E.: *Ber.*, **32**, 2451 (1899).
26. Fischer, E.: *Ber.*, **34**, 433 (1901); **35**, 2660 (1902).
27. Fischer, E.: *Z. physiol. Chem.*, **33**, 151, 177, 412 (1901).
28. Fischer, E.: *Z. physiol. Chem.*, **35**, 70, 221, 227; **35**, 268, 462 (1902).
29. Fischer, E.: *Ber.*, **36**, 2982 (1903); **37**, 2486 (1904); **38**, 605 (1905).
30. Fischer, E., and Abderhalden, E.: *Z. physiol. Chem.*, **46**, 52 (1905); *Ber.*, **39**, 372 (1906); **40**, 3544 (1907).
31. Fischer, E., and Bergell, P.: *Ber.*, **36**, 2592 (1903); **37**, 3103 (1904).
32. Fischer, E., and Fournneau, E.: *Ber.*, **34**, 2868 (1901).

33. Fischer, E., and Otto, E.: *Ber.*, **36**, 2106 (1903).
34. Fischer, E., and Schmitz, W.: *Ber.*, **39**, 351 (1906).
35. Fischer, E., and Warburg, O.: *Ber.*, **38**, 3997 (1905).
36. Fischer, E., and Koenigs, E.: *Ber.*, **37**, 4585 (1905).
37. Folin, O.: *J. Biol. Chem.*, **8**, 9 (1910).
38. Folin, O., and Denis, W.: *J. Biol. Chem.*, **12**, 239 (1912).
39. Folin, O., and Ciocalteu, V.: *J. Biol. Chem.*, **73**, 627 (1927).
40. Folin, O., and Looney, J. M.: *J. Biol. Chem.*, **51**, 421 (1922).
41. Folin, O., and Marenzi, A. D.: *J. Biol. Chem.*, **83**, 89, 103, 109 (1929).
42. Guggenheim, M.: *Z. physiol. Chem.*, **88**, 276 (1913).
43. Harington, C. R., and Barger, G.: *Biochem. J.*, **21**, 169 (1927).
44. Harington, C. R., and Randall, S. S.: *Biochem. J.*, **23**, 373 (1929); **25**, 1917 (1931).
45. Hedin, S. G.: *Z. physiol. Chem.*, **20**, 186; **21**, 155, 297; **22**, 191; **25**, 344 (1895-98).
46. Hofmeister, F.: *Ergebnisse der Physiol.*, **1**, 759 (1902).
47. Hopkins, F. G., and Cole, S. W.: *J. Physiol.*, **27**, 418; **29**, 451 (1902-03).
48. Jones, D. B., Gersdorff, C. E. F., and Moeller, O.: *J. Biol. Chem.*, **62**, 190 (1924).
49. Karrer, P., Schlosser, A., Gränacher, C., and Widmer, R.: *Helv. chim. Acta*, **6**, 1108; **7**, 763; **8**, 203 (1923-25).
50. Kossel, A.: *Z. physiol. Chem.*, **22**, 176 (1896-97).
51. Kossel, A., and Gross, R. E.: *Z. physiol. Chem.*, **135**, 167 (1924).
52. Kossel, A., and Kennaway, E. L.: *Z. physiol. Chem.*, **72**, 486 (1911).
53. Kossel, A., and Patten, A. J.: *Z. physiol. Chem.*, **38**, 39 (1903).
54. Kossel, A., and Staudt, W.: *Z. physiol. Chem.*, **156**, 270 (1926).
55. Lamb, J., and Robson, W.: *Biochem. J.*, **25**, 1231 (1931).
56. Levene, P. A.: *J. Biol. Chem.*, **1**, 45 (1905-06).
57. Looney, J. M.: *J. Biol. Chem.*, **69**, 519 (1926).
58. May, C. E., and Rose, E. R.: *J. Biol. Chem.*, **54**, 214 (1922).
59. Mueller, J. H.: *J. Biol. Chem.*, **56**, 157 (1923).
60. Osborne, T. B., and Co-workers: Numerous papers in *J. Biol. Chem.*
61. Pirie, N. W.: *Biochem. J.*, **27**, 202 (1933).
62. Plimmer, R. H. A., Rosedale, J. L., and Lowndes, J.: *Biochem. J.*, **19**, 1004, 1615; **21**, 247 (1925-27).
63. Pratt, H. E.: *J. Biol. Chem.*, **67**, 351 (1926).
64. Pyman, F. L.: *Trans. Chem. Soc.*, **99**, 1386 (1911).
65. Rimington, C.: *Biochem. J.*, **21**, 1179, 1187 (1927).
66. Schryver, S. B., and Buston, H. W.: *Proc. Roy. Soc. (London)*, **B**, **98**, 58; **99**, 476; **100**, 360; **101**, 519 (1925-27).
67. Sørensen, S. P. L.: *Z. physiol. Chem.*, **44**, 448 (1905).
68. Sullivan, M. X.: *U. S. Public Health Service Reports*, 1030 (1926).
69. Sullivan, M. X.: *U. S. Public Health Service Reports*, **44**, 1421 (1929).
70. Sullivan, M. X., and Hess, W. C.: *U. S. Public Health Service Reports*, **44**, 1599 (1929); Suppl. No. 86 (1930); Suppl. No. 94 (1931).
71. Sullivan, M. X., and Jones, D. B.: *U. S. Public Health Service Reports*, Suppl. No. 82 (1930).
72. Troensegaard, N.: *Z. physiol. Chem.*, **112**, 86 (1921).
73. Van Slyke, D. D.: *J. Biol. Chem.*, **9**, 185; **10**, 15; **12**, 275; **16**, 121, 531; **22**, 281; **23**, 407, 411 (1911-15).
74. Vickery, H. B.: *J. Biol. Chem.*, **71**, 303 (1927).
75. Vickery, H. B., and Block, R. J.: *J. Biol. Chem.*, **93**, 104, 113 (1931).
76. Vickery, H. B., and Leavenworth, C. S.: *J. Biol. Chem.*, **72**, 403; **75**, 115; **76**, 707; **79**, 377 (1927-28).
77. Vickery, H. B., and Osborne, T. B.: *Physiol. Rev.*, **8**, 393 (1928).
78. Vickery, H. B., and Schmidt, C. L. A.: *Chem. Reviews*, **9**, 169 (1931).
79. Vickery, H. B.: *Yale J. Biol. Med.*, **4**, 595 (1932).
80. Wada, M.: *Biochem. Z.*, **224**, 420; **257**, 1 (1930-33).
81. Wheeler, H. L., and Jamieson, G. S.: *Am. Chem. J.*, **33**, 365 (1905).
82. Windus, W., and Marvel, C. S.: *J. Amer. Chem. Soc.*, **53**, 3490 (1931).

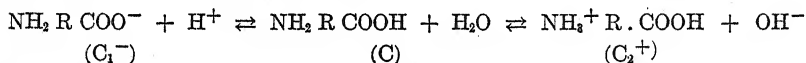
CHAPTER VI

SOME ASPECTS OF THE PHYSICAL CHEMISTRY OF AMINO ACIDS AND PROTEINS

The Amphoteric Character of the Proteins.—A characteristic of the amino acids and proteins is the fact that they contain both acid and basic groups and are therefore amphoteric. In acid solution the ampholyte acts as a base, and in basic solution as an acid. Thus, from the standpoint of the classical dissociation theory, we may write for a simple amino acid such as $\text{NH}_2 \cdot \text{R} \cdot \text{COOH}$ the reactions for its combination with acid and with base



The basic nature is displayed to its full extent in a strongly acid solution and its acid nature in a strongly alkaline solution. In aqueous solution the amino acid behaves like a weak electrolyte. We can represent its acidic and basic properties by means of the equation



Let us assume that the concentration of the amino acid in solution is (C), the concentration of acidic anions is (C_1^-) , and the concentration of basic cations is (C_2^+) . The apparent acid and basic dissociation constants may be designated respectively by K_a' and K_b' . The terms "apparent" acid and basic dissociation constants are used instead of the true dissociation constants for the reason that, in order to determine the true dissociation constant, the activities of the various components of the reaction must be known. In most cases they are not known. The value lies in the vicinity of unity and, in most instances, it can be assumed as being unity. The value for K_a' will then be equal to that of K_a . We shall, therefore, assume $\text{K}_a' = \text{K}_a$, etc. (A discussion of activity coefficients of amino acids is given in another section.)

Now
$$\text{K}_a = \frac{(\text{C}_\text{H}^+) (\text{C}_1^-)}{\text{C}} \quad \text{or} \quad \text{C} = \frac{1}{\text{K}_a} (\text{C}_\text{H}^+) (\text{C}_1^-)$$

and
$$\text{K}_b = \frac{(\text{C}_\text{OH}^-) (\text{C}_2^+)}{\text{C}} \quad \text{or} \quad \text{C} = \frac{1}{\text{K}_b} (\text{C}_\text{OH}^-) (\text{C}_2^+)$$

Since the concentration of undissociated ampholyte is the same for both dissociations,

$$\frac{(C_{H^+})(C_1^-)}{K_a} = \frac{(C_{OH^-})(C_2^+)}{K_b}$$

Substituting $\frac{K_w}{C_{H^+}}$ for C_{OH^-} , and rearranging, we obtain

$$(C_{H^+})^2 = \frac{K_a}{K_b} K_w \frac{(C_2^+)}{(C_1^-)}$$

or

$$C_{H^+} = \left[\frac{K_a}{K_b} K_w \frac{(C_2^+)}{(C_1^-)} \right]^{1/2}$$

The Iso-electric Point.—At the iso-electric point the dissociations of the ampholyte as an acid and as a base are equal and (C_2^+) is equal to (C_1^-) .* The above equation then becomes

$$C_{H^+} = \left(\frac{K_a}{K_b} K_w \right)^{1/2} \text{ or } pI = 1/2 (pK_a - pK_b + pK_w)$$

If a number of acid and basic groups are present in the ampholyte, the equation for the iso-electric point in terms of the dissociation constants of each of the acid and basic groups present becomes

$$I \dagger = \left(\frac{K_{a_1} + K_{a_2} \dots K_{a_n}}{K_{b_1} + K_{b_2} \dots K_{b_n}} \cdot K_w \right)^{1/2}$$

It is obvious that when the values for K_{a_2} , etc., and K_{b_2} , etc., are very small, the effect on the value for I is small and, in most cases, these terms can be neglected. At the iso-electric point the ampholyte is dissociated roughly to the minimum extent, and the solubility, the viscosity, and, in the case of such substances as gelatin, the swelling also, approximate a minimum.

Titration Curves.—The dissociation constants and the iso-electric point of the more soluble amino acids may best be determined by

* Theoretically this assumption is not strictly correct. When $(C_2^+) = (C_1^-)$, then $C_{H^+} = C_{OH^-}$, $K_a = K_b$, and $I = 10^{-7}$ which is not true. Customary usage, however, has defined $I = \left(\frac{K_a}{K_b} K_w \right)^{1/2}$.

† Abramson (Abramson, H. A., *Electrokinetic Phenomena and their Application to Biology and Medicine*) has developed the concept of the iso-electric point statistically. Over a time average the net charge of all of the molecules of the ampholyte must be zero; the instantaneous state of any molecule of the ampholyte is irrelevant. At the iso-electric point

$$\frac{1}{t} \int_0^t dt (A^+) = \frac{1}{t} \int_0^t dt (A^-)$$

If the positive and negative ions in any solution are considered to be respectively of the i and j type, then

$$\frac{1}{t} \int_0^t \sum n_i z_i dt + \frac{1}{t} \int_0^t \sum n_j z_j dt = 0$$

where n is a number, z is the valence, and t = time. This equation is the same as that which defines the iso-electric state of a surface; i. e., that electrical state in which the total sum of all of its positive and negative charges at the surface over a time average is equal to zero.

means of titration curves. To a given amount of the amino acid, varying amounts of NaOH or HCl are added. The solution is brought to the same volume in each case, and the hydrogen ion activity is measured by means of the hydrogen electrode or, in certain instances, by means of the quinhydrone or the glass electrode. The measured potentials may be conveniently reduced to pH by means of the Schmidt and Hoagland tables (Schmidt, C. L. A., and Hoagland, D. R., *Univ. of Calif. Pub. Physiol.*, 5, 23 (1919)), which use the values of 0.336 volt for the N/10 KCl calomel electrode, and 0.283 volt for the normal KCl calomel electrode. The values, 1.005×10^{-14} at 25° C. and 0.58×10^{-14} at 18° C., are used for K_w . A correction is applied to the curve obtained when pH^* is plotted against cubic centimeter of acid or base added by carrying out a water titration blank.

In accord with the simple Henderson-Hasselbalch equation,

$$pH = pK_a + \log \frac{\text{salt}}{\text{acid}} = pK_w - pK_b + \log \frac{\text{base}}{\text{salt}}$$

the reading of pH on the curve at the point of half neutralization with base would represent the value of pK_a , the acid dissociation constant, based on the assumptions (a) that the concentrations of anion and of salt are equal, (b) that the remaining acid is totally undissociated, and (c) that the concentrations of both constituents of the system are equal to their activities. The reading of the point of half neutralization with acid would represent $pK_w - pK_b$; or, at that point,

$$pK_b = pK_w - pH.$$

The value of the titration curve midpoint has been termed by Simms the *titration index*.

Calculation of Dissociation Constants.—The above equations obviously do not give the true values for pK_a and pK_b since (a) the concentration of salt is not equal to the concentration of anion (or cation) on account of hydrolysis; (b) the inherent errors in points in the region of minimum curvature have an unduly large effect on the position of the points in the region of maximum curvature; and (c) the error of plotting is not entirely negligible. The following equation may be used in calculating values for pK_a :

$$pK_a = pH + \log \frac{C_A - C_{Na^+} + C_{OH^-}}{C_{Na^+} - C_{OH^-}}$$

where C_A is the total amino acid concentration, C_{Na^+} and C_{OH^-} are the concentrations of sodium and hydroxyl ions, respectively, in moles per liter. In the case of the primary acid dissociation constant of the dicarboxylic amino acids, hydrolysis becomes negligible as compared to

* In hydrogen electrode measurements, the hydrogen ion activity rather than hydrogen ion concentration is determined. To convert the former into the latter, the activity coefficient must be known. For ordinary purposes it can be assumed to be unity. This is an expedient rather than a suitable approximation. This distinction is brought out by the use of the term paH instead of pH . For practical purposes we shall use the term pH instead of paH .

that of the second acid constant. The assumption that all of the anion comes from the salt is incorrect. The above equation is modified to

$$pK_a = pH + \log \frac{C_A - C_{Na^+} - C_{H^+}}{C_{Na^+} + C_{H^+}}$$

The basic dissociation constant may be calculated from the equation

$$pK_b = pK_w - pH + \log \frac{C_A - C_{Cl^-} + C_{H^+}}{C_{Cl^-} - C_{H^+}}$$

where pK_w is equal to $\log \left(\frac{1}{K_w} \right)$ and at 25° C. is numerically equal to 13.998. Typical titration curves of amino acids are shown in Figs. 1, 2 and 3.

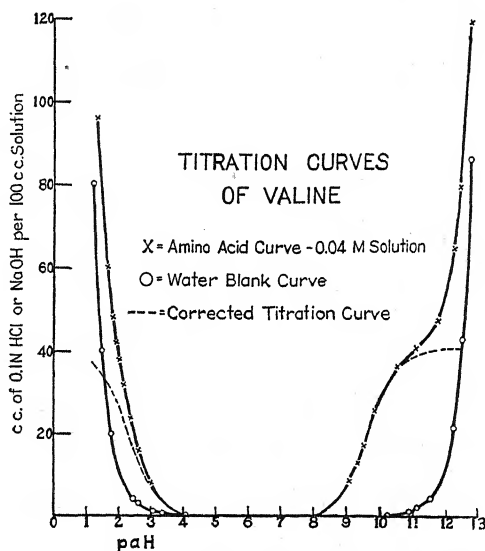
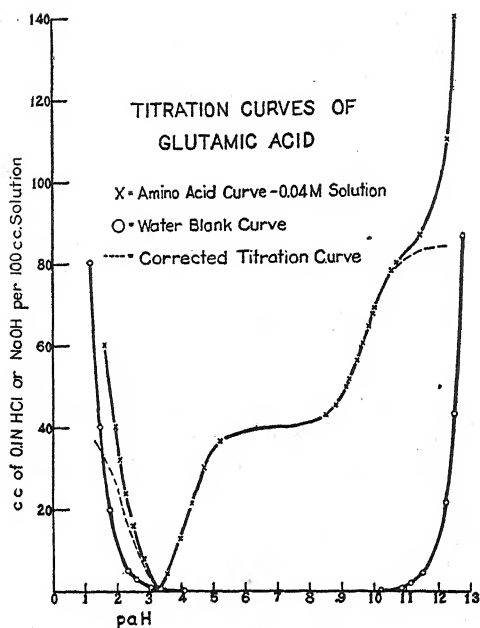
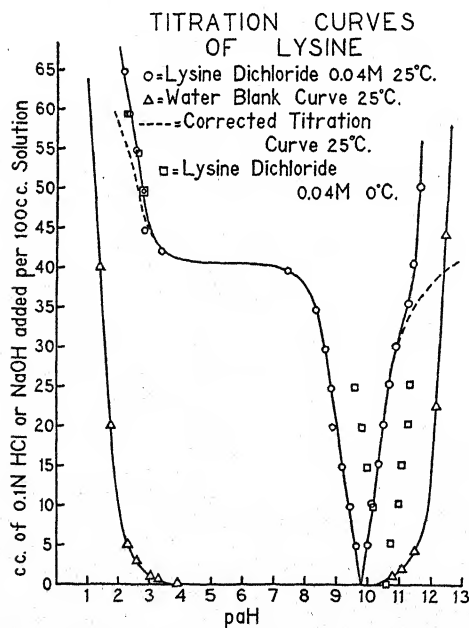


Fig. 1.—(Kirk and Schmidt, *J. Biol. Chem.*)*

Relation Between Solubility and Dissociation.—When the amino acid is only slightly soluble, as in the case of cystine, leucine, tyrosine, and diiodotyrosine, the dissociation constants may be determined from data obtained by estimating the solubility of the amino acid at a constant temperature and varying acidities. This procedure was followed by Merrill, Hitchcock, Sano, and Dalton, Kirk and Schmidt. The relations between solubility and dissociation can be expressed by the following

$$S = S_0 + \frac{S_0 K_a}{C_{H^+}}$$

* When the volume and page number are not given in the reference, see reference section at the end of this chapter.

Fig. 2.—(Kirk and Schmidt, *J. Biol. Chem.*)Fig. 3.—(Schmidt, Kirk, and Appleman, *J. Biol. Chem.*)

and

$$S = S_0 + \frac{S_0 K_b C_{H^+}}{K_w}$$

where S = solubility of the amino acid at the given pH , and S_0 = minimum solubility of the undissociated amino acid. In the case of tyrosine, the complete expression for its solubility at any pH is given by the equation,

$$S = S_0 \left[1 + \frac{K_b C_{H^+}}{K_w} + \frac{K_{a_1}}{C_{H^+}} + \frac{K_{a_1} K_{a_2}}{(C_{H^+})^2} \right]$$

Ester hydrolysis, conductivity, and optical measurements have also been used to estimate dissociation constants of amino acids.

The dissociation constant values given in Table 1 are taken from a compilation by Miyamoto and Schmidt. A more recent compilation has been made by Cohn.

Separating Amino Acids by Electrical Transport.—A practical application of dissociation constant data has been made by Foster and Schmidt in separating amino acids by electrical transport. It will be noted from the curves given in Fig. 4 that at pH 5.5 arginine and lysine

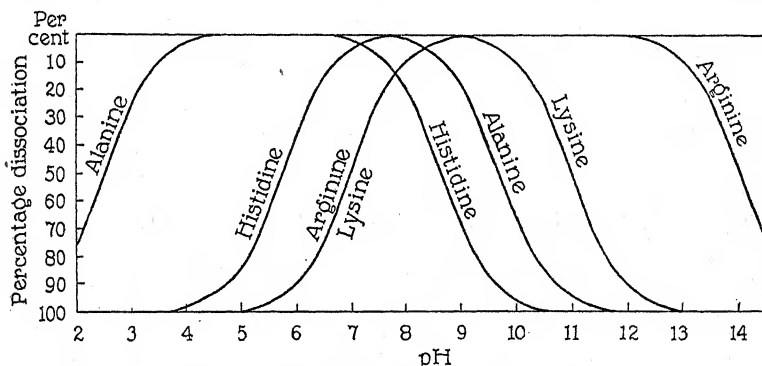


Fig. 4.—(Foster and Schmidt, *J. Biol. Chem.*)

are nearly completely dissociated, while histidine is dissociated to about 50 per cent. The mono amino acids are dissociated only to a slight extent. The dicarboxylic amino acids are combined with base. On passing a direct current through a three-compartment cell in which the mixture of amino acids is contained in the center compartment and water in each of the end compartments, the basic amino acids will migrate to the cathode compartment, the dicarboxylic amino acids will migrate to the anode compartment, while the other amino acids will remain in the center compartment. A separation of histidine from arginine and lysine may be carried out by subjecting the basic amino acids to transport at pH 8.5.

Iso-electric Points of Some Common Proteins.—Since the proteins contain a number of both acidic and basic groups, they may be re-

TABLE 1
THERMODYNAMIC DATA FOR AMINO ACIDS AT 25°¹

| Amino acid. | $K'a$ | $K'b$ | $(\Delta F^\circ)'x_2$ calories | $(\Delta F^\circ)'x_3$ calories | $(\Delta H)'x_3$ calories | $(\Delta S)'x_2$ calories per degree | $(\Delta S)'x_3$ calories per degree |
|------------------------------------|------------------------|------------------------|------------------------------------|------------------------------------|------------------------------|---|---|
| Monosaccharide, monobasic: | | | | | | | |
| Alanine..... | 2.06×10^{-10} | 2.21×10^{-12} | 13,200 | 15,900 | 14,600 | — 4.4 | — 4.4 |
| Cysteine..... | 2.3×10^{-5} | 5.0×10^{-13} | 10,200 | 16,800 | 14,600 | — 5.9 | — 5.9 |
| Glycine..... | 9.6×10^{-10} | 1.5×10^{-12} | 12,300 | 17,500 | 14,600 | — 8.1 | — 8.1 |
| Isoleucine..... | 2.54×10^{-10} | 2.21×10^{-12} | 13,100 | 15,900 | 14,600 | — 4.4 | — 4.4 |
| Leucine..... | 2.09×10^{-10} | 2.39×10^{-12} | 13,200 | 15,900 | 14,600 | — 4.4 | — 4.4 |
| Norleucine..... | 2.5×10^{-10} | 2.3×10^{-12} | 13,100 | 15,900 | 14,600 | — 4.4 | — 4.4 |
| Oxyproline..... | 1.72×10^{-10} | 2.46×10^{-12} | 13,200 | 15,900 | 14,600 | — 4.4 | — 4.4 |
| Phenylalanine..... | 1.86×10^{-10} | 3.32×10^{-12} | 13,300 | 16,500 | 14,600 | — 4.4 | — 4.4 |
| Proline..... | 5.82×10^{-10} | 3.83×10^{-12} | 12,600 | 15,600 | 14,600 | — 4.4 | — 4.4 |
| Serine..... | 2.5×10^{-10} | 1.0×10^{-12} | 14,500 | 16,400 | 14,600 | — 4.4 | — 4.4 |
| Tryptophane..... | 7.08×10^{-10} | 1.62×10^{-12} | 12,800 | 16,100 | 14,600 | — 4.4 | — 4.4 |
| Valine..... | 4.05×10^{-10} | 2.4×10^{-12} | 12,800 | 15,900 | 14,600 | — 4.4 | — 4.4 |
| Monosaccharide, dibasic: | | | | | | | |
| Arginine..... | 3.32×10^{-13} | 1.0×10^{-12} | 17,000 | 6,770 | 12,400 | — 15.5 | — 15.5 |
| Histidine..... | 6.7×10^{-10} | 1.05×10^{-12} | 12,500 | 16,600 | 12,400 | — 10.5 | — 10.5 |
| Lysine..... | 2.95×10^{-11} | 1.01×10^{-12} | 14,400 | 16,800 | 12,400 | — 9.3 | — 9.3 |
| Monobasic, polycyclic: | | | | | | | |
| Aspartic acid..... | 2.24×10^{-10} | 7.51×10^{-13} | 4,980 | 16,500 | 10,500 | — 20.2 | — 20.2 |
| Diiodotyrosine..... | 3.32×10^{-7} | 1.32×10^{-12} | 8,840 | 16,200 | 12,770 | — 11.5 | — 11.5 |
| Dihydroxyphenylalanine..... | 1.51×10^{-10} | 2.31×10^{-12} | 10,700 | 11,800 | 8,790 | — 6.3 | — 6.3 |
| Glutamic acid..... | 2.11×10^{-10} | 1.33×10^{-12} | 13,500 | 15,900 | 12,400 | — 13.5 | — 13.5 |
| β -Hydroxyglutamic acid..... | 2.10×10^{-10} | 1.55×10^{-12} | 15,900 | 16,100 | 12,400 | — 13.5 | — 13.5 |
| Tyrosine..... | 5.62×10^{-10} | 2.12×10^{-12} | 5,800 | 15,900 | 12,400 | — 13.5 | — 13.5 |
| | 2.19×10^{-10} | 1.75×10^{-12} | 13,200 | 16,000 | 12,500 | — 6.7 | — 6.7 |
| | 5.82×10^{-10} | | 5,780 | | | | |
| | 2.76×10^{-10} | | 13,100 | | | | |
| | 6.6×10^{-10} | | 12,500 | | | | |
| | 4.9×10^{-11} | | 14,100 | | | | |

¹ Miyamoto, S. and Schmidt, C. L. A. *J. Biol. Chem.*, **90**, 165 (1931).

² Cysteine is a disaccharide and dibasic amino acid. It is placed in the group of monosaccharide, monobasic amino acids for convenience only.

³ Calculated from the values for $\Delta H'$ at 25° (14).

⁴ G. M. Richardson (*Biochem. J.*, **25**, 917 (1931)) has obtained the following pK' values for β -hydroxyglutamic acid at 25°: 2.09, 4.18 and 9.20. The pK' values for thiohistidine are: 1.84, 8.47, 11.4 (*Biochem. J.*, **27**, 1036 (1933)).

For the determination of dissociation constants without liquid junction potentials see Owen, B. B., *J. Am. Chem. Soc.*, **56**, 24 (1934); Nims, L. F., and Smith, P. K., *J. Biol. Chem.*, **101**, 401 (1933).

garded as multi-acidic and -basic ampholytes. The constants characterizing each set of groups lie so close to each other that usually no sharp breaks occur in the titration curves. A convenient way of determining the iso-electric point of the protein is by studying its path of migration under the influence of a direct current. At the iso-electric point little or no migration occurs. Another method is that of solubility.* Approximate minimum solubility is found at the iso-electric point. The iso-electric points of some of the common proteins are

TABLE 2
ISO-ELECTRIC POINTS OF PROTEINS

| Protein | I |
|---------------|---------|
| Casein | 4.7 |
| Edestin | 6.6 |
| Egg albumin | 4.6 |
| Fibrinogen | 5.0-5.5 |
| Gelatin | 4.7 |
| Globin | 8.1 |
| Hemoglobin | 6.8 |
| Serumalbumin | 5.5 |
| Serumglobulin | 4.4 |

given in Table 2. A discussion of the groups responsible for combination with acid and with base will be taken up in another section.

Jukes and Schmidt, and Neuburger have determined the dissociation constants of certain of the amino acids in ethanol-water mix-

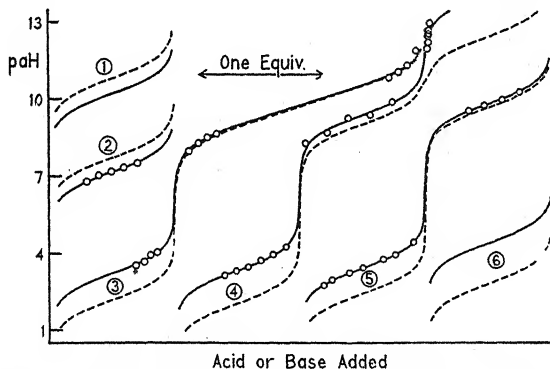


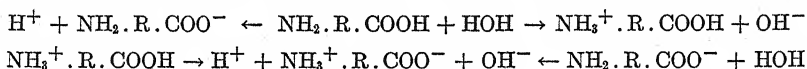
Fig. 5.—Apparent dissociation curves at 25° of ethylamine (Curves 1), glycine ethyl ester (Curves 2), lysine (Curves 3), arginine (Curves 4), glycine (Curves 5), and monochloroacetic acid (Curves 6). The solid lines represent the titration curves in 72 per cent (by volume) of ethyl alcohol and the dashed lines represent the titration curves in water. The scale on the horizontal axis is such that the length given by the arrow represents 1 equivalent of acid or of base added. The points on the curves represent present experimental observations. (Jukes and Schmidt, *J. Biol. Chem.*)

tures. The curves given in Fig. 5 show that the effect of alcohol is to decrease the dissociation of the buffer groups which, in terms of the zwitter ion theory, represent the carboxyl groups of these amino

* Since many proteins possess iso-electric zones, the method of estimating iso-electric points of proteins on the basis of minimum solubility is necessarily not very accurate.

acids, and to increase slightly the pK values corresponding to the α -amino groups. It should be noted in titrating amino acids in ethanol-water mixtures that the effect of the ethanol is primarily upon the phenolphthalein indicator and not upon the amino acids. No simple relationship between the dielectric constant and the pK values of the amino acids appears to exist.

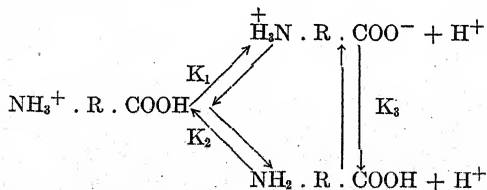
Zwitterions.—Thus far the discussion of amino acid dissociation constants has been based upon the classical theory as developed by Walker and others.* Due to the work of Adams, Bjerrum, Harris, Cohn, Borsook and Macfadyen, Ebert, Schmidt and his coworkers, as well as many others, it is now well established that the amphoteric properties of the proteins and amino acids can be more adequately explained upon the basis that they behave as zwitterions. The relationship of this concept to the classical idea is shown by the following:



The zwitterion in the uncombined state carried both a positive and negative charge and is internally neutralized, while in the classical sense it carried practically no charge. According to the classical view, the effect of adding alkali is to permit the ionization of the carboxyl group which is so weak that it dissociates only in alkaline solutions. Similarly, the addition of acid permits ionization of the amino group. According to the zwitterion hypothesis, the effect of the addition of base is to depress the ionization of the amino group, thus leaving the carboxyl group free to combine with base. Similarly, in acid solution, the dissociation of the carboxyl group is repressed so that the already ionized amino group can form a salt with the acid. If we denote the acid and basic dissociation of the zwitterion, respectively, by K_A and K_B , the relationship between the zwitterion constants and the classical constants is given by

$$K_A = \frac{K_w}{K_b} \text{ and } K_B = \frac{K_w}{K_a}$$

As has been shown by Ebert and by Miyamoto and Schmidt, an equilibrium exists between the zwitterion and the classical form of the amino acid, although the amount of the classical form is negligibly small as compared with that of the zwitterion. The equilibrium may be represented by the equations,



* Literature is given by Kirk and Schmidt, *Univ. of Calif. Pub. Physiol.*, 7, 57 (1929).

(e) Effect on dielectric constant. Water possesses a high dielectric constant. The effect of the addition of an amino acid yields a solution the dielectric constant of which is greater than that of water. This is apparently due to the presence in the amino acid of ionic charges. Zwitterions behave in their saturated aqueous solution more like ideal electrolytes than do ordinary electrolytes. This is due in part to their contribution to the dielectric constant.

(f) Effect of inactivating the amino or the carboxyl group on the value of the dissociation constant. The classical dissociation constant, K_b , of glycine is 2.2×10^{-12} , while for glycine ethyl ester it is 5.37×10^{-7} . The zwitterion constant, K_B , of glycine is 5.55×10^{-5} . The latter value is in better agreement with that for glycine ethyl ester than the classical value for K_b of glycine. A considerable deviation from the zwitterion constant is to be expected since the effect of $-\text{COOR}$ on the dissociation of $-\text{NH}_3^+$ is not the same as the effect of the negatively charged group $-\text{COO}^-$. Addition of formaldehyde to an amino-acid solution converts the amino group into a methylene derivative and thus inactivates it. The latter compound can now be titrated like a simple carboxylic acid. Its dissociation constant is about 1000 times greater than the value for K_a of the amino acid. The new value is more in accord with that of the zwitterion constant of the amino acid.

(g) Wyman and McMeekin have pointed out that the electric moments or polarization of amino acids in water are abnormally large, being primarily explained by their properties as zwitterions. They are approximately the same for all amino acids of a given type and they show, within the limits of experimental error, a linear increase with length of chain separating the acid and basic groups in the molecule. Cohn and his coworkers have pointed out that the apparent molal volumes determined for a number of amino acids agree remarkably well with the volumes calculated according to Traube. The explanation advanced is that the tighter packing of the amino acids depends upon the electrostriction of the solvent molecules resulting from the zwitterion structure.

It is pertinent to point out here that any excess numbers of either acidic or basic groups in any amino acid or protein are not to be regarded as zwitterions. In very few, if any, proteins are the numbers of basic groups exactly equivalent to the numbers of acid groups; usually there is an excess of one or the other. These excess groups do not necessarily come within the scope of zwitterions. Since, in the protein molecule, the positions of the basic and acidic groups are not at present definitely placed, the influence of these respective groups upon each other is still a matter of conjecture. It is very improbable that, if the protein contains an excess number of carboxyl groups, the former will be symmetrically placed with respect to the basic group and thus statistically come under the influence of the latter group. It is more

probable that the positions of the excess basic or acidic groups are such that they can be regarded as being wholly free.

COMBINATION OF PROTEINS WITH ACIDS AND BASES

Proteins as well as amino acids combine with acids and with bases. This can be illustrated by the titration curve of casein in Fig. 6, the

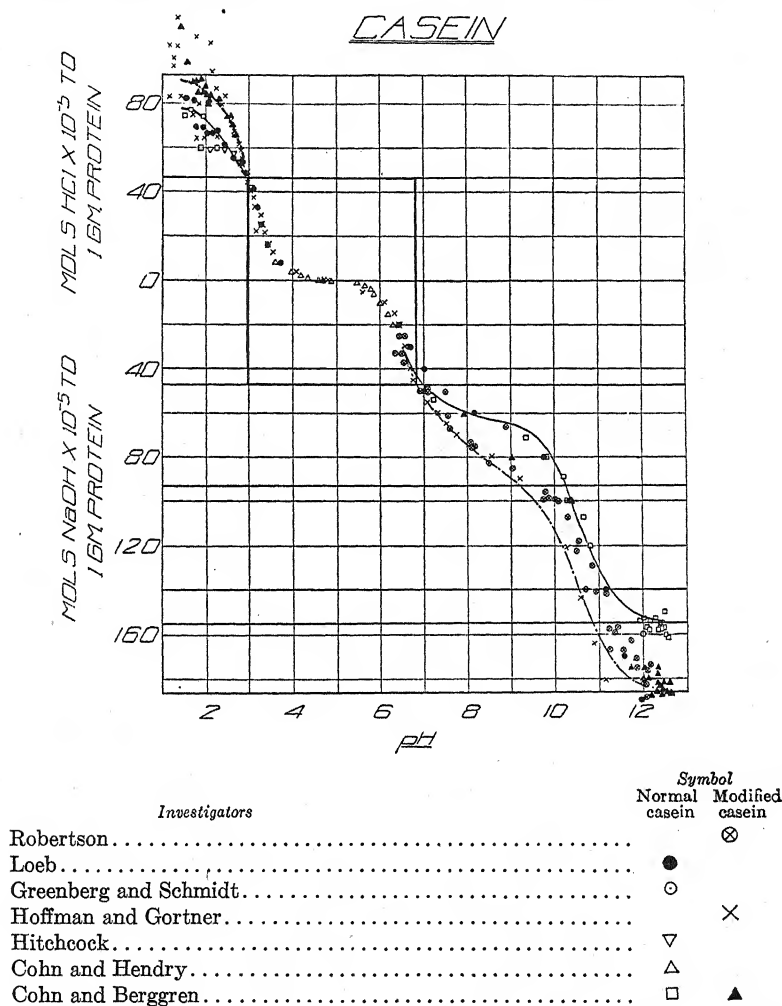


Fig. 6.—Robertson employed potassium hydroxide in his experiments. Casein passes into solution when 1 Gm. is combined with approximately 47×10^{-5} moles of either sodium hydroxide or hydrochloric acid. The acid and base combining curves of normal and modified casein have been constructed on the basis of the following dissociation constants: $pK = 3.00, 7.10, 8.46, \text{ and } 10.55$. (Cohn, *Physiol. Rev.*)

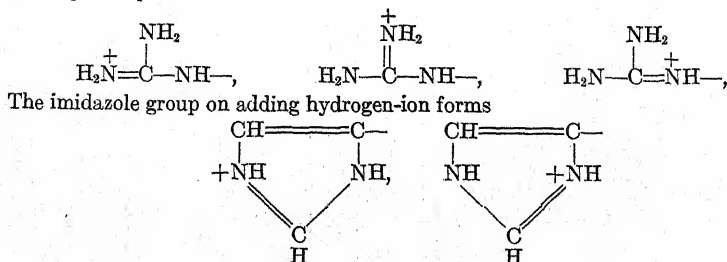
data for which have been collected by Cohn. The capacity to combine with bases and acids depends upon the presence in the molecule

of acidic and basic groups. The chief problem has been to determine the particular groups responsible for the acid and base combining capacity. Proteins react but slowly when treated with nitrous acid. The free amino groups are those which correspond to the epsilon group of lysine. Arginine yields an amount of nitrogen which is equivalent to one half of its total nitrogen when treated for six hours at 25° C. with nitrous acid. The protamin, salmin, reacts likewise, indicating that the guanidine group of arginine is free. The imino group of histidine is likewise free and can combine with acid. Data such as have been collected by Cohn show that a satisfactory correlation exists between the amount of acid with which proteins can combine and the content of arginine, lysine and histidine.* Similarly, the base combining capacity of proteins depends upon the free carboxyl groups contributed by aspartic, glutamic, and hydroxyglutamic acid, plus the hydroxy group which is contributed by tyrosine. From this is subtracted those carboxyl groups of the first three mentioned amino acids which exist as acid amide groups (this can be determined by the amount of ammonia which is set free when the protein is hydrolyzed by acid). Phosphoric acid, which is probably combined in proteins as an ester of serine, tyrosine or hydroxyglutamic acid may also neutralize base.

Stoichiometric Combinations of Proteins with Acids and Bases.—

The titration of protein with acid and base is an equilibrium reaction which necessitates a considerable excess of acid or base to bring the reaction to the desired pH. A somewhat different approach to the problem was made by Chapman, Greenberg and Schmidt and Rawlins and Schmidt. They added either acid or basic dyes to solutions of proteins at a given pH and determined after removal of the protein-dye precipitate the amount of dye left in solution. It was found that the combination between the proteins and the dyes took place in stoichiometric proportions and was dependent upon the number of free acid or basic groups present in the protein molecule. The reaction cannot be considered as one of adsorption. Figure 7 illustrates some of the dye titration curves obtained by them.

* Jukes and Branch (*Science*, 80, 228 (1934)) have pointed out that the acid binding capacity of arginine can be best explained on the assumption that the guanidine group adds H^+ not to the amino but to the imino group. The resulting ion may be represented



Still another approach to the problem was made by Czarnetzky and Schmidt. They treated amino acids and proteins in the solid state

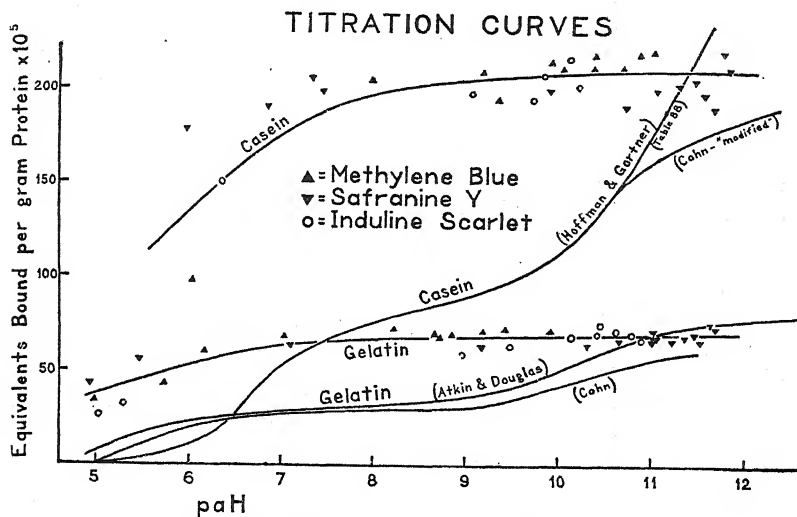


Fig. 7.—(Rawlins and Schmidt, *J. Biol. Chem.*)

with such gases as ammonia or hydrogen chloride and determined the dissociation pressures with increasing amounts of acid or base added.

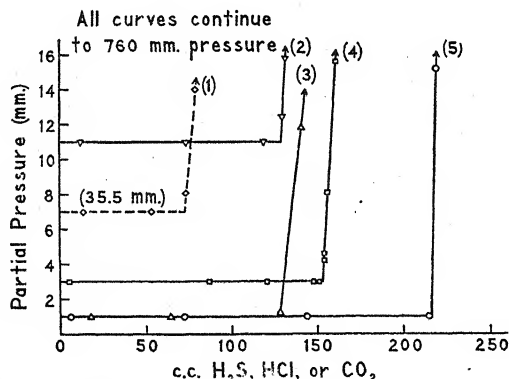


Fig. 8.—Gasometric titration curves showing combination of the basic amino acids with hydrogen chloride, carbon dioxide, and hydrogen sulfide. Curve 1 shows 1 Gm. of histidine with hydrogen sulfide; Curve 2, 1 Gm. of arginine with hydrogen sulfide; Curve 3, 1 Gm. of arginine with carbon dioxide; Curve 4, 1 Gm. of lysine with hydrogen sulfide; Curve 5, 1 Gm. of histidine with hydrogen chloride. The compound which histidine forms with hydrogen sulfide has a dissociation pressure of 35.55 mm. The absolute values on the horizontal portion of Curves 3 and 5 on account of the low pressures are not very accurate. (Czarnetzky and Schmidt, *J. Biol. Chem.*)

On the basis of the phase rule relations, an abrupt change of pressure should take place when complete neutralization of the acid or basic

TABLE 3*

ACID- AND BASE-COMBINING POWER OF CERTAIN PROTEINS

| Protein. | Acid-bound. | Base-bound. | Method. |
|----------------------|---|---|---|
| | <i>moles</i> $\times 10^{-5}$ <i>per Gm.</i> | <i>moles</i> $\times 10^{-5}$ <i>per Gm.</i> | |
| Casein**..... | | 160 | Potentiometric. |
| | 100 | 210 | Dye titration. |
| | | 183 | Potentiometric. |
| | 60, 76, 90 | 155, 136, 140 | " |
| | 59, 72 | | " , recalculated values of Hitchcock and Loeb. |
| | 95 | 180 | Calculated.† |
| | 233 | | Gas titration. |
| Gelatin..... | 284 (230)‡ | 212 | " " present work. |
| | 197 | 150 | Calculated.§ |
| | 103 | | Dye titration. |
| | | 60 | Potentiometric. |
| | 92, 89, 86 | 56, 57 | " , recalculated values of Loeb and Hitchcock. |
| | 89 | 75 | Conductometric titration. |
| | 89 | | Potentiometric. |
| Deaminized gelatin.. | 92 | 70 | " |
| | 107 | 41 | Calculated.† |
| | 302 | | Gas titration. |
| | 342 (306)‡ | 78 | " " , present work. |
| | 317 | 41 | Calculated.§ |
| | 59 | | Dye titration. |
| | 45 | | Potentiometric. |
| Edestin..... | 67 | 41 | Calculated.† |
| | 305 | 80 | Gas titration, present work. |
| | 276 | 41 | Calculated.§ |
| | 157 | 70 | Dye titration. |
| | | 75 | Potentiometric. |
| | | 90 | " |
| | 127 | | " , recalculated values of Kodama and of Hitchcock. |
| Egg albumin..... | 133 | 98 | Calculated.† |
| | 164 | 109 | Gas titration, present work. |
| | 210 | 73 | Calculated.§ |
| | 80 | 80 | Potentiometric, recalculated values of Hitchcock and of Berggren. |
| | 110 | 134 | Potentiometric. |
| | 65 | 81 | Calculated.† |
| | 170 | 162 | Gas titration, present work. |
| Zein..... | 113 | 58 | Calculated.§, |
| | 0 | 28-31 | Potentiometric. |
| | 16 | 61 | Calculated.† |
| | 24 | 56 | Gas titration, present work. |
| | 100 | 28 | Calculated.§ |
| | 22 | 23 | Potentiometric. |
| | 131 | 71 | Calculated.† |
| Globin (ox) ¶..... | 181 | 51 | Gas titration, present work. |
| | 243 | 45 | Calculated.§ |

* Czarnetzky, E. J., and Schmidt, C. L. A.: *J. Biol. Chem.*, 105, 301 (1934).

TABLE 3—Continued

| Protein. | Acid-bound. | Base-bound. | Method. |
|-----------------------------|---|---|------------------------------|
| | <i>moles</i> $\times 10^{-5}$ <i>per Gm.</i> | <i>moles</i> $\times 10^{-5}$ <i>per Gm.</i> | |
| Hemoglobin (ox)¶,*** | 146 | | Calculated.† |
| | 69 | 69 | Potentiometric. |
| | 126 | 68 | Calculated.† |
| | 210 | 48 | Gas titration, present work. |
| | 234 | 43 | Calculated.§ |
| Salmin..... | 496 | | Analysis. |
| | 502 | | Calculated.† |
| | 648 | | Gas titration, present work. |
| | 597 | | Calculated.§ |
| Silk fibroin¶..... | 19 | 55 | "† |
| | 40 | 86 | Gas titration, present work. |
| | 33 | | Calculated.§ |
| Dephosphorized casein¶..... | 280 (230)‡ | 170 | Gas titration, present work |

** The phosphoric acid has not been considered in the calculated values of this and other phosphorus-containing proteins.

† The calculated values for the acid-combining capacity are based on the assumption that arginine, histidine, and lysine each contribute one basic group. The calculated amount of base with which the protein should combine is based on the assumption that glutamic acid, hydroxyglutamic acid, aspartic acid, and tyrosine each contribute one acid group less the amount which is combined as acid amides (based on the amount of ammonia liberated on hydrolysis). The values are those which might be expected when the protein is in aqueous solution.

‡ The values given in parentheses have been corrected for increase of amino nitrogen due to hydrolysis. When no such figures are given, no apparent hydrolysis took place.

§ The calculated values for the acid-combining capacity are based on the assumption that arginine, lysine, proline, oxyproline, and tryptophan each contribute one and histidine contributes two basic groups. The calculated values for the base-combining capacity are based on the assumption that aspartic acid, glutamic acid, and hydroxyglutamic acid each contribute one acid group less the amount which is combined as acid amides (based on the amount of ammonia liberated on hydrolysis). The values are those which might be expected when the solid protein is treated with hydrogen chloride or ammonia gas.

|| On the basis of the analyses published by Calvery, the calculated combining power of egg albumin for acids is 146×10^{-5} and for bases 67×10^{-5} moles per Gm. of protein.

¶ Since the analytical values for the content of amino acids are incomplete or not very reliable, the calculated values are necessarily not very reliable.

*** The calculated values for hemoglobin are based on the content of globin which constitutes about 96 per cent of the hemoglobin molecule. (A recent compilation of acid and base combining-capacities of proteins has been made by Cohn: *Ergebnisse Physiol.*, 33, 781 (1931).)

groups present in the protein molecule has taken place and this was found to be the case. The combining capacities of the solid dry proteins studied could be roughly correlated with the free carboxyl groups. The combining capacities of these proteins for hydrogen chloride are approximately determined by the content of ϵ -amino nitrogen of lysine, the imino nitrogen of tryptophan, the guanidine group of arginine, the tertiary and imino nitrogen groups of histidine, and the tertiary nitrogen of the $-\text{CON}-$ groups (based on the content of proline and oxyproline). It is to be noted that certain groups combine with hydrogen

chloride which do not react with hydrochloric acid in aqueous solution. Typical titration curves of amino acids and proteins with gaseous acids and bases are shown in Figs. 8 and 9. A comparison between the acid- and base-combining capacity of proteins when in the dry state and when in aqueous solution is given in Table 3.

Another illustration of the binding of acid by protein is seen in the experiments of Hitchcock. He added hydrochloric acid to gelatin and measured both the hydrogen and chloride ion activities. The

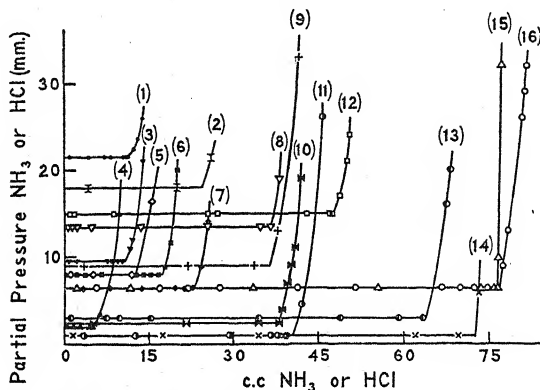


Fig. 9.—Gasometric titration curves showing combination of certain proteins with ammonia or hydrogen chloride. Curve 1 shows 1 Gm. of globin with ammonia; Curve 2, 1 Gm. of edestin with ammonia; Curve 3, 1 Gm. of hemoglobin with ammonia; Curve 4, 1 Gm. of zein with hydrogen chloride; Curve 5, 1 Gm. of zein with ammonia; Curve 6, 1 Gm. of gelatin with ammonia; Curve 7, 0.5 Gm. of hemoglobin, with ammonia; Curve 8, 1 Gm. of crystalline egg albumin with ammonia; Curve 9, 1 Gm. of edestin with hydrogen chloride; Curve 10, 1 Gm. of crystalline egg albumin with hydrogen chloride; Curve 11, 1 Gm. of globin with hydrogen chloride; Curve 12, 1 Gm. of casein with ammonia; Curve 13, 1 Gm. of casein with hydrogen chloride; Curve 14, 0.5 Gm. of salmine with hydrogen chloride; Curve 15, 1 Gm. of gelatin with hydrogen chloride; Curve 16, 1 Gm. of gelatin with hydrogen chloride (reverse titration with the same sample which was used for Curve 15). This reverse titration was made by withdrawing known amounts of hydrochloric acid gas from the system, and plotting the changes in pressure against the number of cubic centimeters of the gas withdrawn. Curves 15 and 16 meet at a pressure of 200 mm. A reverse titration serves as a check on the forward titration. (Czarnetzky and Schmidt, *J. Biol. Chem.*)

curves show that the activity of the hydrogen ion was greatly reduced but there was only a small reduction in the activity of chloride ion. The union of protein and hydrochloric acid is similar to the titration of ammonia with this acid. The degree of ionization of the gelatin chloride was nearly as great as that of the titrating acid. The activity of the chloride ion was not greatly reduced.

DISSOCIATION OF PROTEIN SALTS

When a direct current is passed through a solution of a protein salt the protein migrates either to the anode or cathode depending on which side of the iso-electric point the reaction of the solution is. This

method has been used to determine the iso-electric point, since in the iso-electric condition there is little or no migration of the protein. Electrode measurements have been limited largely to hydrogen and chloride electrodes. This yields us no information about the charge carried by the protein ion. Such information has been obtained by the extensive transport experiments which have been carried out by Greenberg and Schmidt, Miyamoto and Schmidt, and others.

When a protein solution, which is contained in a three-compartment cell, is subjected to electrical transport, a deposit of protein is formed on the platinum wire which serves as anode or cathode. The important thing about this protein deposit is that quantitatively it is proportional to the current passed through the solution; in other words, it obeys Faraday's law of electrodeposition. The electrode deposit is proportional to the current and, within limits, inversely proportional to the amount of acid or base combined with the protein, or to the charge carried by the protein. The relationship can be expressed by the equation,

$$B \times Q = K,$$

where B = the number of cubic centimeters of 0.1 N acid or alkali in combination with each gram of protein, Q is the electrochemical equivalent per millifaraday, and K is a constant. Some values for K are given in Table 4. The experimental results show that the transport

TABLE 4
TRANSPORT NUMBERS OF SOME PROTEIN SOLUTIONS AT 25° C. (AVERAGED VALUES)

| Protein | Dissolved in | pH | K | T Protein | T Cation |
|----------|--------------------------------|----------|------|-----------|----------|
| Casein | LiOH | 6.5-7.2 | 9.95 | 0.485 | 0.48 |
| Casein | NaOH | 6.5-7.0 | 9.55 | 0.425 | 0.62 |
| Casein | KOH | 6.5-9.4 | 9.10 | 0.360 | 0.65 |
| Fibrin A | NaOH | 9.5-10.5 | 5.95 | 0.49 | 0.47 |
| Fibrin B | NaOH | 9.5-10.0 | 9.2 | 0.48 | 0.52 |
| Fibrin C | NaOH | 9.5 | 7.45 | 0.46 | 0.50 |
| Fibrin C | KOH | 10.0 | 8.0 | 0.37 | 0.58 |
| Protein | Dissolved in | pH | K | T Protein | T Anion |
| Fibrin A | HCl | 3.5 | 7.2 | 0.50 | 0.50 |
| Fibrin C | HBr | 3.5 | 4.95 | 0.48 | 0.45 |
| Fibrin C | HNO ₃ | 3.5 | 6.1 | 0.55 | 0.36 |
| Fibrin C | H ₂ PO ₄ | 3.3-2.85 | 4.35 | 0.89 | 0.23 |
| Fibrin C | HCOOH | 3.5 | 4.8 | 1.28 | 0.34 |

(Greenberg, *Trans. Am. Electrochem. Soc.*)

numbers vary inversely with the mobility of the cation used. In a solution of LiOH, casein carries the larger fraction of the current since lithium possesses a small mobility. With ions of higher mobility, this falls off in the order of sodium, potassium, etc.

For a binary electrolyte, the transport numbers of the two constituents are related to each other according to the following equation:

$$\frac{T_{\text{anion}}}{T_{\text{cation}}} = \frac{\Lambda_0 \text{ anion}}{\Lambda_0 \text{ cation}}$$

where Λ_0 is the mobility or equivalent conductivity at infinite dilution. Some mobility values for casein at 30° C. are given in Table 5.

The essential thing about these experiments is the fact that proteins when combined with the alkali metals yield only protein anions and alkali metal cations. In terms of the interionic attraction theory of electrolytes by Bjerrum and Debye and Hückel, it is to be assumed that these protein solutions are completely ionized. When casein is

TABLE 5
MOBILITY OF CASEIN ION AT 30° C.

| Cation Used | T_{Casein} | $\Lambda_0 \text{Cation}$ | $\Lambda_0 \text{Casein}$ |
|-------------|---------------------|---------------------------|---------------------------|
| Lithium | 0.495 | 43.3 | 42.5 |
| Sodium | 0.455 | 56.2 | 46.2 |
| Potassium | 0.365 | 81.4 | 46.5 |
| Rubidium | 0.355 | 84.5 | 45.5 |
| Cesium | 0.335 | 85.3 | 43.0 |

(Greenberg, *Trans. Am. Electrochem. Soc.*)

combined with the alkali earth elements, some of the metallic element migrates to both anode and cathode compartments. As seen from the data given in Table 6, the transport numbers in many cases are greater than unity, indicating the presence in the solution of complex ions. Some of the metallic element migrates in combination with the protein ion. This is illustrated by the equation,

$$M_{\text{a protein}} = x M^{++} + M_{(a-x)} \text{ protein}^{-2x}$$

where M stands for the alkali earth element, a , the concentration, and x , the fraction of protein dissociated. If the mobilities of the ionic species in the solution are known, the amount of cation (or anion) in the form of complex ions can be calculated from the transport number by means of the equation,

$$T_{\text{cation}} = \frac{i u - (1 - i) v}{i(u + v)},$$

where i = fraction of ionized cation, u = mobility of the cation, and v = mobility of the complex ion. Experimentally it was found that when to 1 Gm. of casein 6.75 cc. of $n/10 \text{ Mg(OH)}_2$ were added, 42 per cent of the total alkaline earth element was present as cation. Similarly, when 6.6 cc. of Ca(OH)_2 were added, 33 per cent was cationic, when 6.25 cc. of Sr(OH)_2 were added, 46 per cent was present as cations, and when 7.4 cc. of Ba(OH)_2 were added, 26 per cent of the alkaline earth element was present as cations. Using sodium and barium amalgam electrodes, respectively, in solutions of aspartic and glutamic acids and in sodium and barium caseinates, Kirk and Schmidt showed that solutions of the dicarboxylic amino acids and casein in sodium hydroxide behave as typically strong electrolytes. Within the

pH range studied, the casein ion affected the ionic strength as if it were univalent. Barium caseinate behaved abnormally.

In attempting to explain the formation of complex ions in solutions of protein in the alkaline earth hydroxide, Miyamoto and Schmidt carried out transference and conductivity experiments with both the dicarboxylic amino acids and casein. Again it was established that

TABLE 6

TRANSPORT NUMBERS (AVERAGE VALUES AT 25° C.)

B = cc. 0.1 N alkali per g. of casein.

Q = electrochemical equivalent per millifaraday.

$K = Q \times B$.

| Time | Casein | B approx- imately | pH | Q | K | T _{casein} | T _{cation} |
|------------------------------------|-----------|-------------------------|------|---------------|------|---------------------|---------------------|
| (a) Casein + Mg(OH) ₂ . | | | | | | | |
| Hr. | Per Cent | cc. | | | | | |
| 3.5 | 1.85-2.25 | 6.75 | 7.0 | 1.20 | 8.1 | 0.84 | 0.26 |
| 3 | 2.27 | 7.5 | 7.7 | 0.90 | 6.8 | 0.82 | 0.31 |
| 2.5-3.5 | 1.85-2.55 | 9.5 | 9.3 | 0.74 | 7.1 | 0.74 | 0.40 |
| 3 | 2.28 | 10.45 | 9.8 | 0.66 | 6.9 | 0.71 | 0.27 |
| 3.5 | 1.7-2.2 | 10.9 | 10.0 | 0.56 | 6.2* | 0.79 | 0.20 |
| | | | | Average = 7.2 | | | |
| (b) Casein + Ca(OH) ₂ . | | | | | | | |
| 3.5-4.0 | 1.94-2.57 | 6.6 | 7.2 | 1.15 | 7.6 | 1.00 | (-0.12) |
| 3.25 | 1.77 | 7.7 | 7.7 | 0.99 | 7.6 | 1.05 | (-0.18) |
| 3.5-4.0 | 1.6-2.1 | 9.5 | 9.4 | 0.85 | 8.1 | 0.78 | |
| 3.25 | 1.84 | 11.0 | 10.3 | 0.63 | 7.0 | 0.64 | |
| | | | | Average = 7.5 | | | |
| (c) Casein + Sr(OH) ₂ . | | | | | | | |
| 3.5-4 | 2.13 | 6.25 | 6.8 | 1.31 | 8.2 | 0.73 | 0.24 |
| 3.0-4.25 | 2.3 | 8.0 | 7.6 | 1.02 | 8.2 | 0.75 | 0.25 |
| 3.25 | 2.2 | 9.0 | 8.7 | 0.88 | 7.9 | 0.70 | 0.23 |
| 2.5 | 2.25 | 10.5 | 10.0 | 0.71 | 7.4 | 0.63 | 0.43 |
| | | | | Average = 7.9 | | | |
| (d) Casein + Ba(OH) ₂ . | | | | | | | |
| 3.75 | 1.85 | 7.4 | 7.3 | 0.94 | 7.0 | 1.24 | (-0.07) |
| 4 | 1.85 | 8.0 | 8.0 | 0.80 | 6.4 | 1.06 | (-0.09) |
| 4 | 1.9 | 9.0 | 9.5 | 0.80 | 7.2 | 0.84 | 0.07 |
| 3.25 | 1.81 | 10.5 | 9.9 | 0.70 | 7.3 | 0.77 | 0.31 |
| 2.3 | 1.82 | 13.55 | 10.7 | 0.42 | 5.7* | 0.54 | 0.42 |
| | | | | Average = 7.0 | | | |

* Not used in obtaining average.

(Greenberg, *Trans. Am. Electrochem. Soc.*)

solutions of barium and calcium caseinate yield complex ions. Table 7 gives the mobility data which they obtained. Since a protein such as casein contains a considerable number of free carboxyl groups, it can be considered as a polybasic acid. It is conceivable that the alkaline earth element which is bound to certain of the carboxyl groups is more easily dissociated than that which is bound to the remainder. The net result is step dissociation of the type which takes place in

polybasic acids. If this be true, the nonionized calcium which is bound to certain of the carboxyl groups will, in transference experiments, be carried in a direction opposite to the path of migration of the dissoci-

TABLE 7
COMPARISON OF MOBILITIES OF ANIONS FROM DATA OF CONDUCTIVITIES AND OF TRANSFERENCE NUMBERS*

| Compound | $\Lambda^{\circ}_{\text{compound}}$ | $\Lambda^{\circ}_{\text{anion}(c)}$ | $\Lambda^{\circ}_{\text{anion}(t)}$ | $\Lambda^{\circ}_{\text{anion}(c)} - \Lambda^{\circ}_{\text{anion}(t)}$ |
|-----------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|---|
| Sodium caseinate..... | 100.00 | 44.20 | 45.50 | -1.30 |
| Calcium "..... | 85.20 | 19.10 | † | |
| Sodium dephosphorized caseinate.. | 93.00 | 37.20 | 37.00 | 0.20 |
| Calcium "..... | 92.50 | 26.40 | 11.00 | 15.40 |
| Monosodium aspartate..... | 78.27 | 27.75 | 27.60 | 0.15 |
| Barium diaspertate..... | 91.96 | 27.75 | 27.50 | 0.25 |
| Calcium "..... | 86.57 | 27.75 | 27.70 | 0.05 |
| Monosodium glutamate..... | 75.62 | 25.10 | 25.00 | 0.10 |
| Barium diglutamate..... | 89.31 | 25.10 | 25.00 | 0.10 |
| Calcium "..... | 83.92 | 25.10 | 25.20 | -0.10 |
| Aspartic acid..... | 377.74 | 27.75 | | |
| Glutamic "..... | 375.09 | 25.10 | | |

* The values for the casein and the dephosphorized casein compounds are at 30°. The values for the amino acid compounds are at 25°. $\Lambda^{\circ}_{\text{anion}(c)}$ is calculated from conductivity measurements; $\Lambda^{\circ}_{\text{anion}(t)}$ is calculated from transference experiments. The following values were used for the cation mobilities.

| | At 25° | At 30° |
|--|--------|--------|
| $\Lambda^{\circ}_{\text{Na}^{+}}$ | 50.52 | 55.80 |
| $\Lambda^{\circ}_{\text{Ca}^{2+}}$ | 58.82 | 66.10 |
| $\Lambda^{\circ}_{\text{Ba}^{2+}}$ | 64.21 | |
| $\Lambda^{\circ}_{\text{H}^{+}}$ | 349.99 | 374.80 |

† Since, due to the presence of complex ions in the solution, the transference number of calcium caseinate is greater than unity, the calculated value for $\Lambda^{\circ}_{\text{anion}(t)}$ is negative. This is an irrational value.

(Miyamoto and Schmidt, *J. Biol. Chem.*)

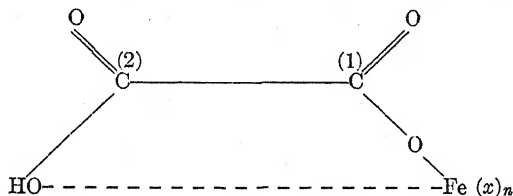
ated calcium ions. The ionization of the alkali metal proteinates must be nearly complete since there is no evidence that these elements form complex ions.

COMBINATION OF PROTEINS WITH HEAVY METALS

The combination of proteins with the heavy metals has been extensively studied. Qualitatively such combinations may be illustrated by the beautiful experiments of Loeb. He immersed gelatin granules in solutions of varying pH and then added silver nitrate to the granules. The excess silver nitrate was removed by washing with cold water, the gelatin was melted, and the tubes of gelatin of varying pH were exposed to sunlight. Only those tubes containing gelatin more alkaline than pH 4.7 turned black. Experiments in which potassium ferrocyanide was used in place of silver nitrate were also carried out. Only those tubes in which the pH was more acid than pH 4.7 turned blue after standing for several days.

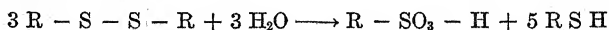
There are comparatively few metallic electrodes which lend themselves to the estimation of the activity of the metallic ion in either protein solutions or solutions of the inorganic salts. Silver is one of those which can be so used. By this method Pauli and Matula found that serum albumin binds 5×10^{-4} gram equivalents per gram of protein. These compounds are but little ionized and therefore lend themselves to therapeutic use.

Indirect methods of determining the combination of heavy metal with proteins also have been used. We may cite the experiments of Smythe and Schmidt. To a standard amount of ammonium thiocyanate they added a mixture of protein and ferric chloride and compared the red color with a similar mixture which did not contain protein. In the case of substances which formed compounds with iron which were less dissociated than ferric thiocyanate, the color of the solution was less intense than that of the standard. Migration experiments were also carried out. The amount of iron which was bound by casein could be roughly correlated with the content of free carboxyl groups resulting from the dicarboxylic amino acids plus the content of phosphoric acid. They suggested that, in the case of the dicarboxylic amino acids, the iron is bound in the following manner:



The iron is very slightly dissociated. If carboxyl group (2) ionizes, then the iron is present in the anionic form. If the pH of the solution is such as to repress entirely the ionization of carboxyl (2), then the only iron that will move with the current is that which dissociates from the above compound and this will be positively charged. By varying the pH it was found possible to change the direction of migration of the iron.

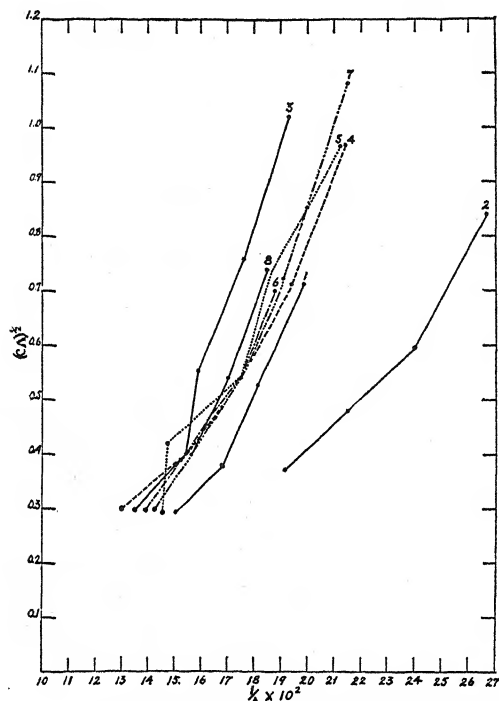
Amino acids also form complexes with heavy metals. Thus Vickery and Gordon have obtained such types of compounds when glycine, glutamic acid, lysine, and other amino acids are treated with HgCl_2 in the presence of alkali. When cystine is treated with either HgSO_4 or Ag_2SO_4 in the presence of dilute sulfuric acid, it is extensively reduced to cysteine with some oxidation to cystic acid according to the equation,



CONDUCTIVITY OF PROTEINS AND AMINO ACIDS

As electrolytes, solutions of the salts of proteins and amino acids conduct the current. Such measurements can only be carried out in

regions of low acidity and alkalinity since otherwise the excess number of hydrogen and hydroxyl ions will carry the larger fraction of the current. According to Greenberg and Schmidt, and Gahl, Greenberg and Schmidt, a straight line is obtained when $(CA)^{1/2}$ is plotted against $\frac{1}{\Lambda}$, where Λ = equivalent conductivity and C = concentration. Such a plot is shown in Fig. 10. A relationship essentially the same



Conductivity Measurements of Sodium Caseinate by various authors, plotted according to Greenberg and Schmidt.

- | | |
|---------------------------|---------------------|
| 1. Pauli and Matula. | pH = 6.3 |
| 2. Robertson. | pH = 6.3 |
| 3. Greenberg and Schmidt. | pH = 6.5 |
| 4. Greenberg and Schmidt. | pH = 6.5 |
| 5. Greenberg and Schmidt. | pH = 6.5 |
| 6. Pauli and Matula. | "slightly alkaline" |
| 7. Laqueur and Sackur. | "slightly alkaline" |
| 8. Greenberg and Schmidt. | pH = 7.5 |

Fig. 10.—(Gahl, Greenberg, and Schmidt, *Univ. Calif. Pub. Physiol.*)

as this was found for certain salts of aspartic and glutamic acid by Miyamoto and Schmidt. With the aid of Onsager's modification of the Debye-Hückel theory for the conductivity of strong electrolytes, they plotted Λ against $\sqrt{n\mu}$ where μ = molecular concentration and n is a number (2 or 3). Such curves show deviations from a straight line in a manner similar to that found by Onsanger for strong electrolytes. The curves are shown in Figs. 11, 12 and 13. In Table 8 are given value for α calculated according to Onsager's equation.

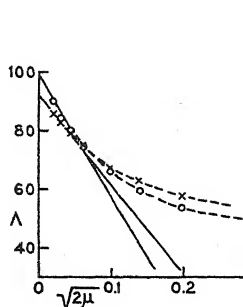


Fig. 11.

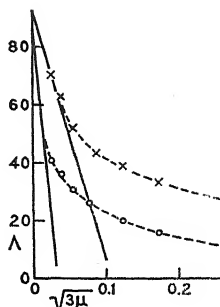


Fig. 12.

Fig. 11.—Conductivity of sodium caseinate, o, and of sodium dephosphorized caseinate, x, plotted according to Onsager's equation.

Fig. 12.—Conductivity of calcium caseinate, o, and of calcium dephosphorized caseinate, x, plotted according to Onsager's equation. (Miyamoto and Schmidt, *J. Biol. Chem.*)

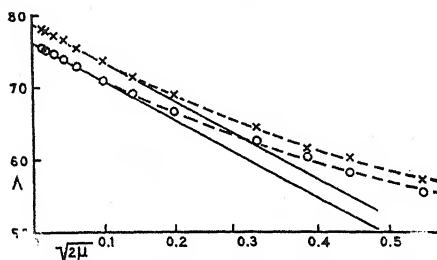


Fig. 13.—Conductivity of sodium aspartate, x, and of sodium glutamate, o, plotted according to Onsager's equation. (Miyamoto and Schmidt, *J. Bio Chem.*)

TABLE 8

EXPERIMENTAL AND CALCULATED VALUES FOR α IN ONSAGER'S EQUATION

| Salts | Λ^0 | $\alpha_{\text{experimental}}$ | $\alpha_{\text{theoretical}}$ | $\alpha_{\text{experimental}}$ minus $\alpha_{\text{theoretical}}$ |
|--------------------------------------|-------------|--------------------------------|-------------------------------|--|
| Sodium chloride..... | 108.89 | 54.69 | 53.00 | +1.70 |
| Monosodium glutamate..... | 75.62 | 50.80 | 47.72 | +3.08 |
| “ aspartate..... | 78.27 | 51.70 | 48.15 | +3.55 |
| Barium nitrate..... | 116.95 | 92.80 | 86.90 | +5.90 |
| “ diaspertate..... | 91.96 | 93.30 | 94.45 | -1.15 |
| “ diglutamate..... | 89.31 | 95.30 | 93.45 | +1.65 |
| Calcium chloride..... | 116.69 | 88.00 | 86.10 | +1.90 |
| “ diaspertate..... | 86.57 | 90.80 | 91.25 | -0.45 |
| “ diglutamate..... | 83.92 | 89.80 | 90.75 | -0.95 |
| Sodium caseinate..... | 100.00 | 425.0 | 51.6 | +374.4 |
| “ dephosphorized case- inate..... | 93.00 | 290.0 | 50.5 | +239.5 |
| Calcium caseinate..... | 85.20 | 2750.0 | 87.0 | +2663.0 |
| “ dephosphorized case- inate..... | 92.50 | 870.0 | 92.3 | +777.7 |

$$\Lambda = \Lambda^0 - \alpha \sqrt{I}$$

(Miyamoto and Schmidt, *J. Biol. Chem.*)

$$\Lambda = \Lambda_0 - a \sqrt{\Gamma}$$

where Λ = molar conductivity, Γ = twice the ionic strength = $(Z_1 + Z_2 - - -)\mu$, a = a constant, μ = molecular concentration, and Z_1 and Z_2 = valences of anions and cations. The reason for the large difference between the calculated and experimental values for a in the case of casein is not clear. It may be due to extensive aggregation of the casein molecules. It is significant that the mobility of the casein ion (see Table 7) is exceptionally high. The temperature gradient of the casein ion mobility is likewise very high (3 mhos per degree centigrade). The gradient for the sodium ion is 1.05 mhos, for potassium 1.32 mhos, and for cesium 1.44 mhos per degree. The mobility values of the amino acids are less than those of the casein ions or even of the acetate ions.

Conductivity measurements of amino-acid solutions, as predicted by Walker, show that these ampholytes do not obey the Ostwald dilution law. The tendency of the values for K_Λ is to become constant only at extremely high dilutions, whereas weak acids, such as acetic, obey the law in fairly high (0.13 M) concentrations.

According to McBain and Dawson, the mobility (in terms of ionic conductivity) of glycine in water from diffusion measurements is 39.2 and the mobility is 40.6 ($39.2 \div 0.965 = 40.6$).

ACTIVITY COEFFICIENTS

A few activity data relating to amino acids have been reported. These have been chiefly determined from freezing point measurements. Reference to the work of Fränkel, Lewis, and Cann, and the equations which apply to their data, are given in the section on thermodynamics.

Hoskins, Randall and Schmidt carried out freezing point measurements with aspartic and glutamic acids and their monosodium salts. These data were used to calculate activity coefficients. They indicated that (a) the undissociated part of glutamic and aspartic acids in solution exists to a considerable extent as neutral aggregates, and (b) the ionized part of the monosodium salts of these acids in solution exists to a slight extent as ionic aggregates or micelles. Values for the activity coefficients, γ_u , of the undissociated parts of aspartic and glutamic acid corresponding to various concentrations are given in Table 9, while values for γ , the "overall" activity coefficient, are

TABLE 9*
ACTIVITY COEFFICIENTS OF THE UNDISSOCIATED PART OF ASPARTIC
AND OF GLUTAMIC ACIDS

| Substance analyzed. | m_u | γ_u |
|---------------------|-------|------------|
| Aspartic acid. | 0.01 | 0.606 |
| | 0.02 | 0.516 |
| | 0.03 | 0.471 |
| Glutamic acid. | 0.01 | 0.702 |
| | 0.02 | 0.644 |
| | 0.03 | 0.615 |

* Hoskins, W. M., Randall, M., and Schmidt, C. L. A.: *J. Biol. Chem.*, **88**, 215 (1930).

TABLE 10*
ACTIVITY COEFFICIENTS OF ASPARTIC AND GLUTAMIC ACIDS
AND THEIR MONOSODIUM SALTS AT 0°

| <i>m</i> | <i>Aspartic acid.</i> | γ |
|------------------------------|-----------------------|----------|
| 0.0025..... | | 0.202 |
| 0.0050..... | | 0.139 |
| 0.0075..... | | 0.111 |
| 0.010..... | | 0.097 |
| 0.015..... | | 0.076 |
| 0.020..... | | 0.065 |
| 0.025..... | | 0.057 |
| 0.030..... | | 0.051 |
| <i>Glutamic acid.</i> | | |
| 0.0025..... | | 0.169 |
| 0.0050..... | | 0.116 |
| 0.0075..... | | 0.092 |
| 0.01..... | | 0.080 |
| 0.015..... | | 0.064 |
| 0.020..... | | 0.055 |
| 0.025..... | | 0.048 |
| 0.030..... | | 0.044 |
| <i>Monosodium aspartate.</i> | | |
| 0.01..... | | 0.830 |
| 0.02..... | | 0.777 |
| 0.03..... | | 0.750 |
| 0.04..... | | 0.731 |
| 0.05..... | | 0.720 |
| 0.06..... | | 0.711 |
| <i>Monosodium glutamate.</i> | | |
| 0.01..... | | 0.817 |
| 0.02..... | | 0.760 |
| 0.03..... | | 0.726 |
| 0.04..... | | 0.703 |
| 0.05..... | | 0.686 |
| 0.06..... | | 0.673 |

* Hoskins, W. M., Randall, M., and Schmidt, C. L. A.: *J. Biol. Chem.*, **88**, 215 (1930).

given in Table 10. For purposes of calculating γ , the following equation is used:

$$\log \gamma = -\frac{j}{2.3} - \frac{2}{2.3} \int_0^m \frac{j_2}{m^{1/2}} dm^{1/2}$$

where m = molality, and $j = 1 - \frac{\theta}{\nu \lambda m}$; θ = the freezing point lowering, and ν = the number of ion molecules formed by the dissociation of a molecule. For purposes of calculation, values for the integral are obtained by plotting $\frac{j}{m^{1/2}}$ (the freezing point function) against $m^{1/2}$, as shown in Fig. 14, and the area below the curve is graphically integrated. This is algebraically increased by an area which represents the difference between the measured curve and the theoretical curve ($\Delta \frac{j}{m^{1/2}}$ is plotted against $m^{1/2}$).

The values for γ for aspartic and for glutamic acid are much smaller than the values for α obtained from conductivity measurements. The value for $\frac{\gamma}{\alpha}$ is about 0.75 for aspartic, and 0.55 for glutamic acid.

The activity data obtained from freezing point measurements apply only to the low temperatures at which they were obtained. The activity for other temperatures may be calculated with the aid of the equation,

$$d \ln (a/a^\circ)/dT = - (\bar{H} - \bar{H}^\circ)/RT^2$$

where a = activity. Values for $(\bar{H} - \bar{H}^\circ)$ for some of the amino acids are given by Zittle and Schmidt (see section on thermodynamics).

By the use of the equation giving the activity coefficient of an amino-acid solution as a function of concentration (see section on thermodynamics), and using the activity coefficient data which are available, the activity coefficients of a saturated solution of glycine at

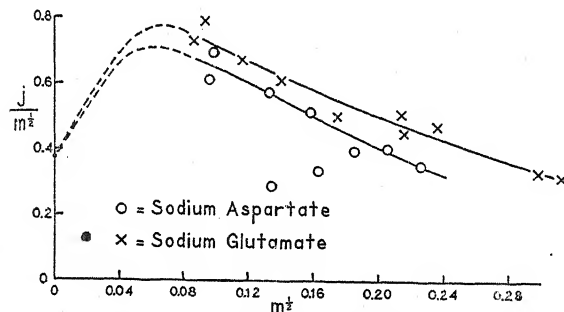


Fig. 14.—Freezing point function for the monosodium salts of aspartic and glutamic acids. (Hoskins, Randall, and Schmidt, *J. Biol. Chem.*)

25° C. are respectively 0.624, 0.663 and 0.894. By using $(\bar{H} - \bar{H}^\circ)$ as a function of temperature in the equation which is given in the previous paragraph, the activity coefficient of the saturated solution at 25° C. is found to increase 13 per cent for a change in temperature from -3° to 25° C. When $(\bar{H} - \bar{H}^\circ)$ is used as a constant, the increase is only 8 per cent. The reason for this discrepancy is not yet apparent.

Some idea of the magnitude of the activity coefficient may be obtained by comparing the heats of solution calculated from solubility measurements, on the basis of ideal behavior, with those obtained by direct measurements. Calculations have given the approximate value 0.9 for the activity coefficient of a saturated solution of glycine at 25° C.

On the basis of Hoskins, Randall and Schmidt's activity coefficient data for aspartic and glutamic acids, Miyamoto and Schmidt have shown, in the case of those two amino acids, that no serious error is

made by assuming that the values for the apparent dissociation constants are equal to the true dissociation constants.

Adair has attempted to obtain activity data of certain salts of hemoglobin on the basis of osmotic pressure measurements. The assumption was made that the average valence of the protein ions is -8.5 . His calculations were made with the aid of the equation,

$$RT \ln f_{ps} m_p = RT \ln a_{ps} = \int v_s dp + \text{constant},$$

where m_p = gram moles of protein salt per liter of solvent, and $f_{ps} = a_{ps}/m_p$ (a coefficient which approaches unity at low protein concentrations). These data are given in Table 11.

TABLE 11*

THE ACTIVITY COEFFICIENTS OF A SALT OF HEMOGLOBIN AND ITS IONS

Conditions: Temperature, 0° ; pressure, 760 mm.; composition of "solvent," 0.10 mole of KCl, 0.0613 mole of Na_2HPO_4 , 0.00533 mole of KH_2PO_4 per liter. Composition of protein salt, $\text{Hb}(\text{Na} + \text{K})_{8.5}$. Valence of protein ion, $n_p = -8.5$. The activity coefficients of the sodium and potassium ions are approximately equal to 0.7.

| Hb. per liter of solvent, mole, m_p . | Act. of protein salt, $\dagger a_{ps}$. | Act. coeff. of salt, f_{ps} . | Act. coeff. of ion, f_p . |
|---|--|---------------------------------|-----------------------------|
| 0.0002 | 0.0002 | 1.05 | 1.03 |
| 0.0010 | 0.0014 | 1.40 | 1.3 |
| 0.0020 | 0.0040 | 2.0 | 1.7 |
| 0.0030 | 0.0090 | 3.0 | 2.3 |
| 0.0040 | 0.0200 | 5.0 | 3.6 |

* Adair, G. S.: *J. Am. Chem. Soc.*, **51**, 696 (1929).

\dagger The determination of a_{ps} by the evaluation of the integral in the above formula may be facilitated by application of the empirical formulas discussed in *Proc. Roy. Soc.*, London, 120A, 573 (1928). In dilute solutions the formula $p(v_s - b) = RT$ can be applied and by integration $RT \ln a_{ps} = \int v_s dp = RT \ln p + bp$. Over the range of pressures from 12 to 120 mm., a formula with two empirical constants can be applied, $p(v_s - 107.5) = 1.09 RT$.

VALENCE OF PROTEIN IONS

It is not a simple matter to determine the valence of the ions which complex substances, such as the proteins, yield. Adair has attempted to obtain values for several proteins from membrane potential measurements. He employed the equation,

$$n_p = 0.00425 MJ (E/C_v)_0,$$

where n_p = mean valence of the protein ions, M = molecular weight of the protein, J = sum of the concentrations of the ions in the dialysate multiplied by the square of the valences, and $(E/C_v)_0$ = limiting values of the ratio at $C_v \rightarrow 0$, as determined by extrapolation. A

rough value for the valence of edestin was found to be 24 on the assumption that the activity coefficient of H^+ is the same as that of the Cl^- ions. The approximate mean value for serum albumin was -25.4 .

PROPERTIES OF PROTEINS DEPENDING ON ACIDITY OR ALKALINITY

Such properties of protein salts as swelling, viscosity, osmotic pressure, and membrane potential depend on the pH of the solution. To understand these phenomena it is best to consider first the Donnan membrane equilibrium. Let us consider a solution of protein chloride separated from a hydrochloric acid solution by a membrane impermeable to the protein ion, but permeable to the ions formed from hydrochloric acid, as shown in part (a) of Fig. 15. The equivalent concentration of protein salt, which is assumed to be completely ionized, is designated by Z .

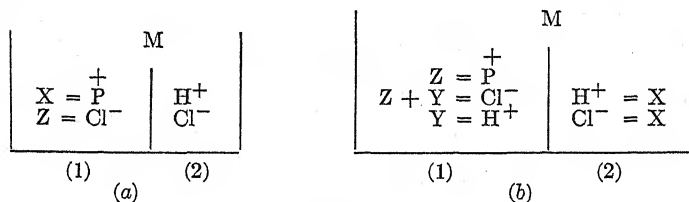


Fig. 15.

Since the membrane is permeable to hydrochloric acid, this substance will pass from compartment (2) to (1) until equilibrium is established, as shown in (b) of Fig. 15. The condition for the establishment of equilibrium between the two sides of the membrane is that the product of the activity (X) of the hydrogen and of the chloride ions in compartment (2) shall be equal to the same product in compartment (1). At equilibrium the free energy change $(\Delta F)_{P,T}$ is zero. The change in free energy which is required to transport reversibly and isothermally dn moles of H^+ from (2) to (1) is equivalent to the energy gained by the corresponding reversible and isothermal transport of dn moles of Cl^- . The free energy exchange is given by the equation,

$$(\delta F)_{P,T} = \delta n RT \log \frac{(H^+)_2}{(H^+)_1} + \delta n RT \log \frac{(Cl^-)_2}{(Cl^-)_1} = 0$$

or

$$\frac{(H^+)_2}{(H^+)_1} = \frac{(Cl^-)_1}{(Cl^-)_2}$$

If the symbols denoting concentrations which are given in Fig. 15 (b) are used, then

$$\frac{X}{Y} = \frac{Y + Z}{X} = \lambda$$

A more rigorous relationship is given when activities instead of con-

concentrations are used. For ordinary purposes no serious error is made when the values for concentrations are taken to be the same as those of the activities. Since the product of the concentration of hydrogen and chloride ions on the two sides of the membrane is equal,

$$X > Y \text{ and } Y + Z > X$$

As a result of the unequal distribution of hydrogen ions (or of chloride ions) on the two sides of the membrane, a potential is established which may be measured by an electrode which is reversible to the particular ion in question. If the electrodes are connected in such

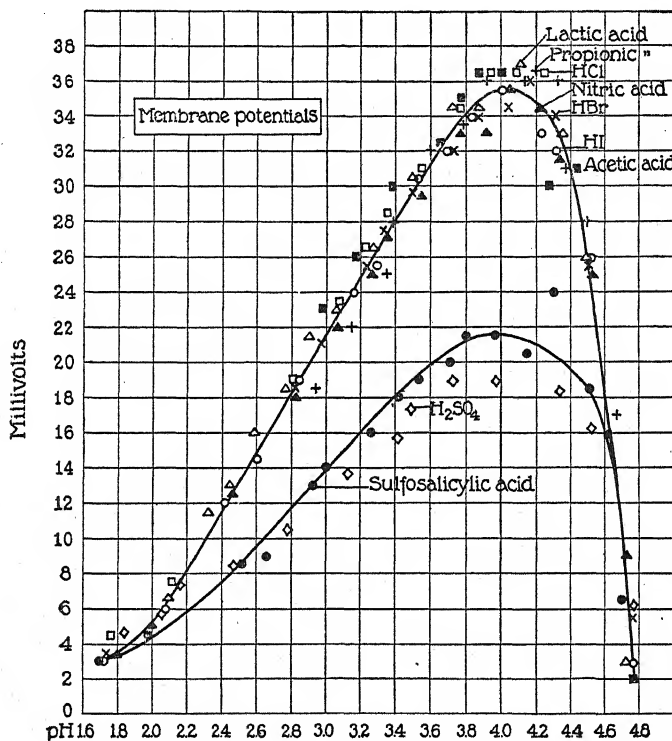


Fig. 16.—(Loeb, *Proteins and the Theory of Colloidal Behavior.*)

a manner that there is no liquid junction potential, the potential difference between the two sides of the membrane with respect to hydrogen ions (or of chloride ions) is given by the equation,

$$E = \frac{RT}{F} \ln \frac{(H^+)_2}{(H^+)_1} = \frac{RT}{F} \ln \frac{(Cl^-)_1}{(Cl^-)_2}$$

where E = potential, R is the gas law constant (8.316 joules per degree), T = absolute temperature, F = Faraday constant (96,500 coulombs), \ln = natural logarithms. If the measurements are carried

out at 25° C. and the pH term is used, the above expression becomes

$$E = 0.05915 (pH_1 - pH_2).$$

The above equations have been developed for univalent electrolytes. The same relations hold for multivalent electrolytes. The generalized expression is

$$\lambda = \sqrt[n]{\frac{(B^{n+})_2}{(B^{n+})_1}} = \sqrt[m]{\frac{(A^{m-})_1}{(A^{m-})_2}}$$

where B = a cation of valency n , A = an anion of valency m , and λ = the Donnan distribution ratio. When $\lambda = 1$, its logarithm is zero, and there will be no membrane potential. In proteins this is true when the pH value corresponds to the iso-electric point. When the value for λ is either greater or less than unity, the difference of the membrane potentials will have a finite value but with corresponding opposite signs depending upon whether the pH is on the acid or the alkaline side of the iso-electric point. The curves given in Fig. 16 illustrate the influence of the pH and the valence of the ion on the membrane potential of gelatin solutions.* Neutral salts depress the magnitude of the membrane potential at any given pH , the amount being determined by the valence of the ion of opposite charge to the charge on the protein.

OSMOTIC PRESSURE

A very practical illustration of the influence of the Donnan membrane equilibrium is found in the measurement of the osmotic pressures of protein solutions. It will be assumed that van't Hoff's law,

$$P = RTC$$

where P = osmotic pressure, R = gas constant, T = absolute temperature, and C = molar concentration, is applicable to protein solutions of moderate concentrations, although, as pointed out by Adair and Burk and Greenberg, this is true for very dilute protein solutions only. A rigorous thermodynamical treatment of osmotic pressure of protein systems is given by Adair. In the protein system represented in Fig. 15, the molar concentration of protein will be designated by a . The factors which influence the osmotic pressure on side (1) are: $(H^+) = Y$ and $(Cl^-) = Y + Z$. The counter osmotic pressure on side (2), due to the HCl , is determined by the value of $2X$. The total osmotic pressure of the system will be

$$P = RT(2Y + Z - 2X + a) = RTe + RTa$$

where $e = 2Y + Z - 2X$. The term e only is affected by the Donnan equilibrium; the term a is a function of the molecular weight of the protein. Since the molecular weight of most proteins is very large and

* For a more complete discussion, see Loeb, J.: *Proteins and the Theory of Colloidal Behavior*, 2nd edition, p. 188, and Hitchcock, D. I.: *J. Gen. Physiol.*, 9, 97 (1925-26).

therefore the osmotic pressure is small, the term, $R T a$, can be neglected and the osmotic pressure at any temperature becomes a function of e only.*

Since most proteins are insoluble in water at their respective iso-electric points, the determination of molecular weights in this state is not feasible. Burk and Greenberg overcame this difficulty by determining the osmotic pressures of a number of proteins in 6.6 M urea solution in which solvent the proteins are soluble although, in the case of hemoglobin and egg albumin, denaturation takes place. The influence of acidity on osmotic pressure was determined by the addi-

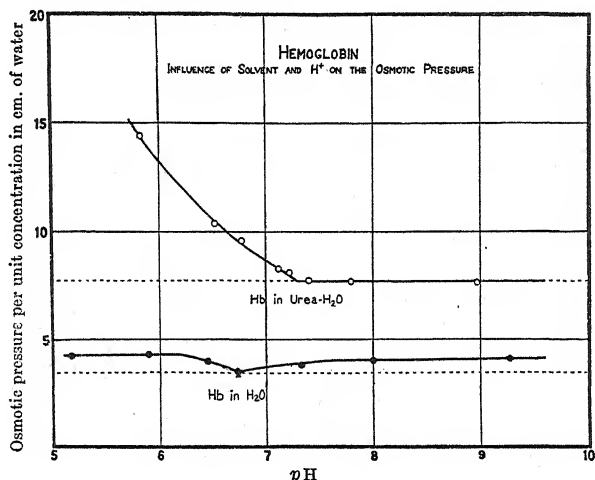


Fig. 17.—The osmotic pressure of hemoglobin in different solvents. Dotted lines represent the minimum osmotic pressure in each solvent, corresponding to that of hemoglobin without the presence of a Donnan membrane equilibrium. (Burk and Greenberg, *J. Biol. Chem.*)

tion of buffer mixtures to the urea solution. The hydrogen ion activities of such buffered solutions had to be determined, since it was found that the hydrogen ion activities of the buffer were not the same in urea solutions as in water. The iso-electric points (or zones) of the proteins were likewise not the same in urea solutions as in water. Figure 17 is illustrative of the effect of pH on hemoglobin solutions.

* The osmotic pressure of gelatin, which is a minimum at pH 4.7, rises with addition of acid to pH 3.4, and then drops upon the addition of more acid. This is due to the Donnan effect. When a little HCl is added to a solution of iso-electric gelatin, gelatin chloride is formed, and some free acid remains, due to hydrolytic dissociation. Both the concentration of Cl^- in combination with gelatin (Z) and the concentration of Cl^- of the free HCl (Y) increase, but (Z) increases at first more rapidly than (Y). The excess of ions inside over that of the ions outside increases until the greater part of the gelatin is transformed into gelatin chloride. The excess of crystalloidal ions inside over those outside reaches a maximum. After that (Z) increases comparatively less than (Y) so that finally (Z) becomes negligible in comparison with (Y). When sufficient acid has been added, the Donnan "correction," $2 Y + Z - 2 X$, becomes zero and the observed osmotic pressure will correspond to that at the iso-electric point.

The osmotic pressure of casein, edestin, and hemoglobin was found to increase to more than would be expected if it were proportional to the increase in concentration, indicating deviation from the laws of perfect solution. The osmotic pressure curves were corrected for the proportionality deviation by use of the equation,

$$C = \frac{100 C'}{100 - hC'}$$

where C' = the measured concentration, C = corrected concentration, and h is a constant whose value is 2.8. The following molecular weights were obtained: Casein, 33,600; edestin, 49,500; hemoglobin, 34,300; egg albumin, 36,000. The molecular weight of hemoglobin in urea solution is one half of that in water, while in glycerol solution, in which it is not denatured, the molecular weight was the same as in water. Burk obtained the value 74,600 for the molecular weight of horse serum albumin in aqueous buffered solution, and 73,000 for the denatured product when dissolved in urea solution.

ULTRAFILTRATION

Greenberg and Greenberg have shown that the Donnan equilibrium must be considered in ultrafiltering solutions containing proteins. A simple illustration is the system which contains sodium caseinate and sodium chloride. If a small amount of liquid is obtained from a large volume of this system, such that the concentration of protein is not significantly altered, the concentration of sodium chloride in the ultrafiltrate is given by the equation,

$$X = [Y_o(Y_o + Z_o)]^{1/2}$$

where X = concentration of NaCl in the ultrafiltrate, Z_o = equivalent concentration of sodium combined with the casein, and Y_o is the concentration of NaCl in the original system. For a bivalent salt such as Na_2SO_4 , the relations are

$$X = [Y_o(Y_o + Z_o)]^{1/3}$$

VISCOSITY

Einstein has formulated the viscosity relationships which exist in a solution at constant temperature by means of the equation,

$$\eta = \eta_o(1 + 2.5\phi)$$

where η_o = viscosity of water, η = viscosity of the solution, and ϕ = fraction of the volume occupied by the solute in the volume of the solution. This equation is only valid when the values for ϕ are small. When the relative volume is large, as in certain protein solutions, the modified equation, suggested by Arrhenius, is more applicable:

$$\log \eta - \log \eta_o = \delta\phi$$

where δ = a constant.

Viscosity measurements carried out on solutions of egg albumin agree fairly well with the Einstein equation, while gelatin solutions follow the Arrhenius formulation. The difference appears to be due to the volumes occupied respectively by these proteins. In the case of gelatin, the protein probably forms submicroscopic particles which are capable of occluding water. They not only occupy larger volumes than egg albumin, but are also subject to the influences of the Donnan effect. The influence of pH , like that observed in osmotic pressure measurements, is to increase the viscosity. This reaches a maximum at about pH 2.8 and then falls rapidly. The viscosity of a suspension of gelatin particles is greater than when the gelatin is in solution. Neutral salts depress the viscosity.

For such cases as are influenced by an electric double layer, which may increase the apparent volume of the particles by introducing an additional resistance due to electrokinetic effects, Smoluchowski has suggested the formulation,

$$\eta = \eta_0 \left[1 + f(\varphi) \left\{ \left(1 + \left(\frac{\zeta D}{2\pi} \right)^2 \frac{R}{\eta_0 r^2} \right) \right\} \right]$$

where ζ = electrokinetic potential, D = dielectric constant, and r = radius of the particles. The relations demanded by this equation have not yet been quantitatively established.

SWELLING

Procter, Procter and Wilson, and Wilson and Wilson have developed the ideas relating to the swelling of gelatin in the presence of hydrogen ions and the relation of the Donnan equilibrium thereto. Their theory postulates that the protein ions constituting a jelly such as gelatin chloride cannot diffuse. They exert no appreciable osmotic pressure, while the combined chloride ions are retained in the jelly by the attractive forces of the gelatin and exert an osmotic pressure. The difference in the diffusibility of the two ions in gelatin chloride gives rise to a Donnan equilibrium which is expressed by

$$X^2 = Y(Y + Z)$$

where the symbols have the same meaning as previously given. The osmotic force, e , for the absorption of water by the gel can be expressed by

$$e = 2Y + Z - 2X$$

On the assumption that gelatin chloride is highly ionizable, Wilson and Wilson showed that

$$V(K + Y)(CV + 2\sqrt{CVY}) - Y = 0$$

where V = increase in volume (cubic centimeter per milliequivalent of gelatin), C = constant corresponding to the modulus of elasticity of gelatin, and K = a constant defined by (gelatin) $(H^+) = K(\text{gelatin ion})$.

REFRACTIVITY

Craig and Schmidt have studied the influence of concentration of the amino acid or the protein as well as of pH upon the refractive index of such solutions with the aid of the interferometer. The influence of amino-acid concentration on the value of $\frac{N}{C}$, where N = refractive index and C = concentration in equivalents per liter, is graphically represented in Fig. 18. The refractive index, moreover, is a function of the amount of acid or base added, or, in other words, upon the amount of ionization of the solute. Figure 18 shows the

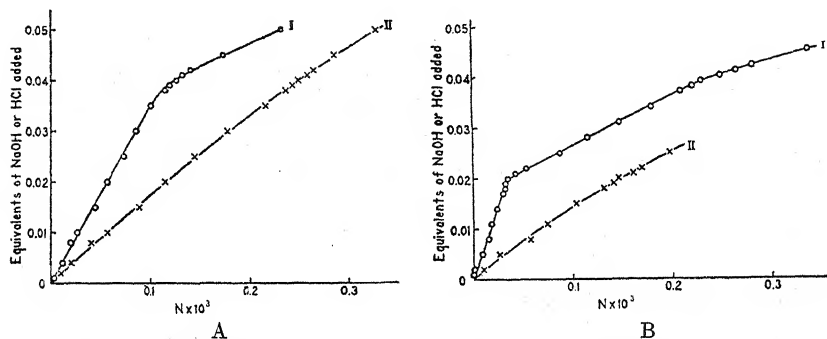


Fig. 18.—A, Titration of glycine. I with NaOH. II with HCl. The curves are theoretical. The points are experimental. In Curve II there is no evidence of a break, and even the point of maximum curvature is very difficult to ascertain. B, Titration of arginine-mono-hydrochloride. I with NaOH. II with HCl. The break at 0.02 equivalent of NaOH in Curve I is the transition from the acid salt to the free acid. From this point on, the curve is theoretical. Curve II is also theoretical, and is very similar to the previous curves for the weak base. Curve II embraces only the second K_b for arginine. (Craig and Schmidt, *Australian J. Exp. Biol. Med. Sci.*)

titration curves of two amino acids. The same factors which govern the change of refractive indices of solutions of amino acids on addition of strong acid or base are also operative in such proteins as edestin and serum albumin.

OPTICAL ACTIVITY

The optical rotation in solutions of optically active amino acids, as shown by Lutz and Jirgenson, is dependent upon the degree of dissociation or the pH of the solution. This is likewise demonstrated in the case of proteins by the data of Almquist and Greenberg which are graphically represented in Fig. 19. Attempts to correlate the optical rotation with the content of the free basic or acidic groups were only partially successful, since probably other factors are also concerned. Thus, for example, the formation of an acid amide on the distal carboxyl group of a dicarboxylic amino acid as asparagine, completely eliminates the rotatory effect and almost no rotational change is shown in alkaline solutions.

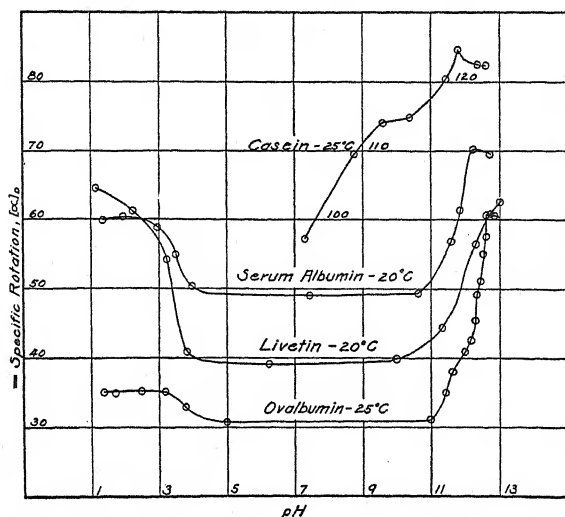


Fig. 19.—Effect of pH on change of specific rotation of several proteins. (Almquist and Greenberg, *J. Biol. Chem.*)

MOLECULAR WEIGHTS OF PROTEINS

Some of the methods, such as boiling and freezing points, which are applicable for the estimation of the molecular weights of small molecules, cannot very well be used to estimate the molecular weights of proteins. Two general methods have been employed: (a) Stoichiometric. The basis for this is the content of heavy metal as in hemoglobin, or the amino-acid content of the protein, and (b) physical chemical behavior of protein solutions.

As an example of the first, we may consider a simple compound such as glutamic acid. The content of nitrogen was determined to be 9.5 per cent. The molecular weight in this instance, on the assumption that the molecule contains only one atom of nitrogen, is

$$\frac{\text{Per cent of N found}}{100 \text{ Gm. of compound}} = \frac{9.5}{100} = \frac{14}{X} = 147 \text{ (minimum molecular weight)}$$

If the compound contained n atoms of nitrogen, the molecular weight would be $n \times 147$. In order to evaluate n , additional data, such as freezing point depression, are necessary. If dissociation of the compound occurs, a serious error is introduced unless the percentage of dissociation is known. In the case of a peptide which contains cystine, both the nitrogen and sulphur content can be used for purposes of calculation. Let us assume that the molecular weight based on nitrogen content is 80, and on the content of sulphur, 200. The data indicate that there are present 2 atoms of sulphur, 5 of nitrogen, and that the molecular weight of the compound is 400. In choosing an element or an amino acid for the basis of molecular weight calculations, it is necessary that the value for n be small. If sulphur-containing amino

acids other than cystine, or if both cystine and cysteine are present in the protein, or, if the cystine content is high, the sulphur content may be of but little use in calculating minimum molecular weights. In such cases the content of other amino acids such as tyrosine or several amino acids when present in small amounts may be used for purposes of calculation.

In the case of the respiratory proteins, oxygen-combining capacity, together with content of heavy metal (since a direct correlation exists between these two), can be used to calculate minimum molecular weights. Combining capacity of the protein for acid and base has likewise been employed. This, however, is less accurate since it may involve a large error in determining these quantities.

Osmotic pressure measurements are extensively used to determine molecular weights of proteins. This necessitates the choice of an appropriate solvent in which the iso-electric protein is soluble, or a correction for the Donnan effect when the protein is combined with acid or base. Both of these aspects are discussed elsewhere. A rigorous treatment of the factors involved in the determination of osmotic pressure of hemoglobin in salt solutions is given by Adair (*Proc. Roy. Soc. (London)*, 120A, 573 (1928)).

Svedberg has made extensive use of the ultracentrifuge for the determination of the molecular weights of proteins. Two methods of approach are afforded. The first depends on the sedimentation equilibrium. As the protein settles under the influence of the centrifugal field, a concentration is eventually reached at the bottom of the cell of such a magnitude that the back diffusion of the particles just balances the sedimentation caused by the centrifugal force. The molecular weight, M , is calculated with the aid of the equation,

$$M = \frac{2 RT \ln (C_2/C_1)}{(1 - V\rho) \omega^2(x_2^2 - x_1^2)}$$

where ω = angular velocity of the centrifuge, ρ = density of the solvent, V = partial specific volume of solute, and C_1 and C_2 are concentrations at the respective points X_1 and X_2 from the center of rotation.

The second method, that of determining molecular weights from sedimentation velocity data in the ultracentrifuge, requires considerably less time and has, in recent times, come into more extensive use. The specific sedimentation velocity, s , which is a constant for every molecular species at a given temperature and for a given solvent, is defined by

$$s = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x} \cdot \frac{\eta}{\eta_0} \cdot \frac{1 - V\rho_0}{1 - V\rho}$$

and the molecular weight is given by

$$M = \frac{RT s}{D(1 - V\rho)}$$

where η and ρ are, respectively, viscosity and density of the solvent, η_0 and ρ_0 = same terms for water at 20° C., D = diffusion constant which may be determined from the concentration of the protein at a given distance from the meniscus after a given time, and $\frac{dx}{dt}$ = sedimentation velocity. The original articles should be consulted for further details.

A surprising finding of Svedberg is that most of the molecular weights of the proteins studied are multiples of 34,500. It is not yet certain whether this fact indicates that the proteins are built up of units of this size, either as micelles or as integral chemically combined units, or, since the molecular weights are large numbers, they are approximately divisible by 34,500. The following are a few of Svedberg's results: Egg albumin, 34,500; hemoglobin, 68,000; hemocyanin, 5,000,000; phycocyan, 105,900; serum globulin, 104,000; phycoerythrin, 207,000.

SOLUBILITIES AND PARTIAL MOLAL VOLUMES OF AMINO ACIDS

Dalton and Schmidt, and Dunn and his coworkers have, in recent times, determined the effect of temperature on the solubility of amino acids in water. In Table 12 are summarized the published and unpublished coefficients of solubility equations of the former workers. They have also determined the densities of solutions of the more soluble amino acids. From these data it is possible to compute the partial molal volumes with the aid of the relation

$$\phi = (V - n_1 v_1) / n_2$$

where ϕ = apparent volume occupied by one mole of the solute, n_1 = number of moles of solvent, n_2 = number of moles of solute, and V = total volume. If the weight of water is 1000 Gm., $n_1 v_1$ becomes 1002.94 cc. at 25° C., and n_2 becomes the molality, m . Values for V are obtained by dividing the sum of the weight of the solvent, 1000 Gm., and the weight of the solute that it contains by the density. If ϕ is plotted against $\log m$, the slope of the curve may be designated as s . This value, s , is divided by 2.303 and to the quotient is added the corresponding value of ϕ at that point. This sum can be demonstrated to be equal to the partial molal volume of the solute, \bar{v}_2 . Now

$$V = n_1 \bar{v}_1 + n_2 \bar{v}_2$$

where \bar{v}_1 = partial molal volume of the solvent, and \bar{v}_2 = partial molal volume of solute. Then since \bar{v}_2 , n_2 and n_1 are also known, and since V can be calculated from the weight of a solution and its density, then \bar{v}_1 can be calculated. In Table 13 are summarized the values so obtained. The calculated values agree in most cases quite well with those which were computed with the aid of the empirical atomic volumes given by Traube. A similar agreement has been obtained by Cohn and his coworkers.

TABLE 12*
COEFFICIENTS OF SOLUBILITY EQUATIONS† OF CERTAIN AMINO ACIDS

| Substance. (1) | a_1 (2) | $b_1 \times 10^2$ (3) | $c_1 \times 10^5$ (4) | a_2 (5) | a_3 (6) | $b_2 \times 10^2$ (7) | $c_2 \times 10^5$ (8) | a_4 (9) | $b_3 \times 10^2$ (10) | $c_3 \times 10^5$ (11) | Maximum deviation.‡ per cent | Mean deviation.§ per cent |
|---------------------------------|--------------|--------------------------|--------------------------|--------------|--------------|--------------------------|--------------------------|--------------|---------------------------|---------------------------|------------------------------------|---------------------------------|
| <i>D</i> -Alanine..... | 2.1048 | 0.4669 | | 0.1551 | -2.5792 | 1.075 | | -6.5150 | 1.037 | | -0.40 | +0.25 |
| <i>L</i> -Alanine..... | 2.0830 | 0.5603 | | 0.1333 | -3.2190 | 1.291 | | -7.1317 | 1.245 | | -0.25 | +0.66 |
| <i>L</i> -Asparagine..... | 0.9289 | 0.2319 | -4.981 | -1.2475 | -25.9584 | 1.291 | -11.47 | -30.2463 | 1.179 | -11.84 | +2.47 | +1.00 |
| <i>D</i> -Aspartic acid..... | 0.3104 | 0.519 | | -1.8047 | -13.7113 | 3.499 | | -17.7370 | 3.502 | | +2.08 | |
| <i>L</i> -Aspartic acid..... | 0.4181 | 2.016 | -4.999 | -1.7060 | -25.9118 | 10.93 | -11.51 | -29.2797 | 10.98 | -11.61 | +1.62 | +0.76 |
| <i>L</i> -Cysteine..... | -1.299 | 1.357 | | -3.680 | -18.043 | 3.125 | | -21.023 | 3.125 | | +1.01 | +1.01 |
| <i>D</i> -Dioxy-L-tyrosine..... | -0.690 | 1.92 | | -3.326 | -19.745 | 4.42 | | -23.761 | 4.43 | | +3.15 | +3.15 |
| <i>D</i> -Glutamic acid..... | 0.6331 | 1.613 | | -1.6345 | -13.9054 | 3.714 | | -17.9095 | 3.709 | | +3.15 | +1.36 |
| <i>D</i> -Glutamine..... | 0.9317 | 1.523 | | -1.2359 | -12.4244 | 3.507 | | -16.4071 | 3.495 | | +7.90 | +3.34 |
| Glycine..... | 2.1516 | 1.087 | -4.114 | 0.2762 | -13.2619 | 7.676 | | -17.8976 | 8.171 | -10.50 | -2.30 | +0.92 |
| <i>D</i> -Isoleucine..... | 1.5787 | 0.07862 | | -0.3889 | 2.9651 | 3.081 | 9.473 | -1.3913 | 3.020 | 5.866 | -2.72 | +1.26 |
| <i>D</i> -Leucine..... | 1.2616 | 0.2512 | 3.794 | -0.8560 | 2.9651 | 3.081 | 8.736 | -1.3913 | 3.020 | 5.866 | -2.72 | +1.26 |
| <i>L</i> -Leucine..... | 1.5561 | 0.02233 | 3.727 | -0.7615 | 3.4260 | 4.683 | 8.582 | -1.3913 | 3.020 | 5.866 | -2.72 | +1.26 |
| <i>D</i> -Methionine..... | 0.9013 | 0.2635 | 4.591 | -1.2163 | -3.45073 | 5.167 | 10.87 | -0.7252 | 3.814 | 7.198 | -1.61 | +0.79 |
| <i>D</i> -Methionine..... | 1.2974 | 1.108 | | -0.9140 | -11.1632 | 4.086 | | -15.2093 | 4.111 | -2.871 | +1.69 | +0.70 |
| <i>L</i> -Phenylalanine..... | 1.2974 | 1.108 | | -0.9140 | -11.1632 | 4.086 | | -15.2093 | 4.111 | -2.871 | +1.69 | +0.70 |
| <i>D</i> -Proline..... | 0.9245 | 0.4522 | 3.109 | 0.1275 | -6.3293 | 3.923 | 7.533 | -10.5103 | 3.601 | 7.340 | -2.40 | +0.66 |
| <i>D</i> -Nucleoside..... | 0.9245 | 0.4522 | 3.109 | 0.1275 | -6.3293 | 3.923 | 7.533 | -10.5103 | 3.601 | 7.340 | -2.40 | +0.66 |
| <i>D</i> -Phenylalanine..... | 0.9088 | 0.5252 | 3.140 | -1.2192 | -0.7183 | 3.739 | 7.299 | -6.2067 | 2.461 | 6.803 | +1.42 | +1.04 |
| <i>D</i> -Serine..... | 1.3432 | 1.520 | -8.548 | -0.6782 | -17.2153 | 7.963 | | -27.0593 | 8.134 | | +1.63 | +0.80 |
| Taurine..... | 1.5945 | 1.916 | -8.50 | -0.5029 | -27.8015 | 15.10 | -19.57 | -32.1293 | 15.33 | -20.07 | +1.98 | +1.41 |
| <i>L</i> -Tryptophan..... | 0.9166 | 0.4834 | 2.988 | -1.3942 | -11.3824 | 4.872 | 6.881 | -15.3098 | 4.869 | 6.879 | +1.41 | +1.43 |
| <i>L</i> -Tyrosine..... | -0.708 | 1.46 | | -2.966 | -10.799 | 3.36 | | -20.082 | 3.37 | | +3.40 | +0.54 |
| <i>D</i> -Valine..... | 1.7749 | 0.2389 | 2.607 | -0.2966 | -2.2921 | -2.729 | 6.003 | -1.7417 | -2.705 | 5.923 | +1.31 | |

* Dalton, J. B., and Schmidt, C. L. A., *J. Biol. Chem.*, 103, 549 (1935).

† Solubility equations:

$$\log S = a_1 + b_1 I + c_1 I^2$$

$$\log m = a_2 + b_2 I + c_2 I^2$$

$$\ln \gamma_2 = a_3 + b_3 I + c_3 I^2$$

$$\ln \gamma_2 = a_4 + b_4 I + c_4 I^2$$

The constants for Equation 1 are given in Columns 2 to 4. The constants for Equation 3 are given in Columns 6 to 8. The constants for Equation 4 are given in Columns 9 to 11.

‡ Maximum deviation of the observed from the calculated values.

§ Calculated from the formula, mean deviation = $(\Sigma D^2/n)^{1/2}$.

For solubility values of amino acids in alcohol-water mixtures etc., see Cohn, E. J., McMeekin, T. L., Edsall, J. T., and Wear, J. H., *J. Am. Chem. Soc.*, 56, 2270 (1934).

(1)
(2)
(3)
(4)

TABLE 13*
 PARTIAL MOLAL VOLUMES OF AMINO ACIDS†

| Amino acid. | v_1 † | v_2 ‡ | Traube. | m ‡ |
|--|---------|---------|---------|---------|
| <i>d</i> -Alanine..... | 18.069 | 60.89 | 58.8 | 0.20 |
| <i>dl</i> -Alanine..... | 18.069 | 60.85 | 58.8 | 0.20 |
| <i>l</i> -Asparagine H ₂ O..... | 18.069 | 97.0 | | 0.1843 |
| <i>l</i> -Aspartic acid..... | 18.069 | 76.9 | 74.7 | 0.03699 |
| <i>dl</i> -Aspartic..... | 18.069 | 75.2 | 74.7 | 0.0548 |
| <i>d</i> -Glutamic acid..... | 18.069 | 90.8 | 90.7 | 0.06449 |
| <i>dl</i> -Glutamic acid..... | 18.069 | 90.5 | 90.7 | 0.1466 |
| Glycine..... | 18.069 | 43.77 | 42.7 | 0.20 |
| <i>d</i> -Isoleucine..... | 18.069 | 105.5 | 107.1 | 0.3461 |
| <i>dl</i> -Isoleucine..... | 18.069 | 106.3 | 107.1 | 0.1647 |
| <i>l</i> -Leucine..... | 18.069 | 107.1 | 107.1 | 0.1828 |
| <i>dl</i> -Leucine..... | 18.069 | 110.0 | 107.1 | 0.07848 |
| <i>dl</i> -Methionine..... | 18.069 | 106.43 | 106.5 | 0.23 |
| <i>dl</i> -Norleucine..... | 18.069 | 110.0 | 107.1 | 0.09076 |
| <i>l</i> -Phenylalanine..... | 18.069 | 128.7 | 130.6 | 0.1794 |
| <i>dl</i> -Phenylalanine..... | 18.069 | 123.3 | 130.6 | 0.08379 |
| <i>dl</i> -Serine..... | 18.069 | 61.62 | 61.1 | 0.473 |
| Taurine..... | 18.069 | 72.27 | | 0.87 |
| <i>dl</i> -Valine..... | 18.069 | 91.06 | 91.1 | 0.6814 |

* Dalton, J. B., and Schmidt, C. L. A.: *J. Biol. Chem.*, **103**, 549 (1933).

† See also Cohn, E. J., McMeekin, T. L., Edsall, J. T., and Blanchard, M. H.: *J. Am. Chem. Soc.*, **56**, 784 and 2270 (1934).

‡ v_1 = partial molal volume of the solvent; v_2 = partial molal volume of the solute; m = molality in moles per 1000 Gm. of water.

THERMODYNAMICS

The solubility values enable us to calculate the heats of solution of the amino acids. According to Schröder's modification, the van't Hoff equation is given by

$$\delta \ln N_2 / \delta T = \Delta H / RT^2$$

where N_2 = mole fraction of solute, T = absolute temperature, R = gas constant, and ΔH = differential heat of solution. Since probably not all of the amino acids form perfect solutions, it is necessary to

apply the relationships as formulated by Brönsted. His equations may be written

$$\Delta H = RT^2 \left(\frac{\delta \ln m}{\delta T} + \frac{\delta \ln \gamma}{\delta m} \cdot \frac{dm}{dT} \right)$$

for unionized solutes, and

$$\Delta H = 2 RT^2 \left(\frac{\delta \ln m}{\delta T} + \frac{\delta \ln \gamma}{\delta m} \cdot \frac{dm}{dT} \right)$$

for ionized solutes. In the above equations, γ = activity coefficient, and m = molality in moles per 1000 Gm. of water.

By way of illustration, we shall show how the heat of solution of glycine may be calculated with the aid of the above equations. Fränkel, Lewis, and Cann have determined the activity coefficients of glycine. The data may be expressed in the form of equations

$$\ln \gamma = -0.1814 \ln m - 0.2538 \text{ (Fränkel)}$$

$$\ln \gamma = -0.1616 \ln m - 0.2165 \text{ (Lewis)}$$

$$\ln \gamma = -0.0607 \ln m - 0.0396 \text{ (Cann)}$$

By modifying the equation for unionized solutes to

$$\Delta H = RT^2 \left(\frac{\delta \ln m}{\delta T} \right) \left(1 + \frac{\delta \ln \gamma}{\delta \ln m} \right)$$

the activity equations, together with the solubility equation, can be differentiated and substituted directly. Using Fränkel's data, the equations for $\ln m$ (Table 12), and the above equation

$$\Delta H = RT^2 (7.676 \times 10^{-2} - 1.895 \times 10^{-4} T) (1 - 0.1814)$$

At 25° C., $\Delta H_{298} = 2930$ calories per mole. Lewis' data give 3010, and the data of Cann 3370 calories per mole. Since at higher temperatures the solution tends to become more nearly perfect, at $T = 298^\circ \text{ K.}$, the true differential heat of solution should lie between the values indicated above and $\Delta H_{298} = 3590$ calories. Calorimetric measurements indicate that $\Delta H_{298} = 3530$ calories when one mole of glycine is dissolved in an infinite amount of a solution of glycine saturated at 25° C.

Table 14 gives the comparison of the values which have been calculated from solubility measurements with those which have been measured by Zittle and Schmidt.

Zittle and Schmidt have also obtained values for the specific heats of glycine, *d,l*-alanine, and *d,l*-valine. From these values, the total heat capacity, \bar{C}_p , of solutions of these amino acids is obtained. The tangent of the curve obtained when the total heat capacity values are plotted against the molality values gives the partial molal heat capacity of the solute. The partial molal heat capacity of the solvent can be calculated with the aid of the following:

$$n_1 d \bar{C}_{p_1} + n_2 d \bar{C}_{p_2} = 0$$

$$\bar{C}_{p_1} = - \int_{n_2=0}^{n_2=m} \frac{n_2}{n_1} d \bar{C}_{p_2} + \bar{C}_{p_2}$$

TABLE 14*

DIFFERENTIAL HEATS OF SOLUTION (CALORIES PER MOLE) OF CERTAIN AMINO ACIDS

All values are for 298° K. unless otherwise stated.

| Substance. | ΔH ideal. | ΔH corrected.† | ΔH calorimetric. |
|-------------------------------------|-------------------|------------------------|--------------------------------------|
| <i>d</i> -Alanine..... | 1830 | 1830 | |
| <i>dl</i> -Alanine..... | 2200 | 2200 | 2250 \pm 20 |
| | 2050 (288°) | 2050 (288°) | 2020 (288°) |
| <i>d</i> -Arginine..... | | | < 1000 \pm 75 (for 1.0 mole) |
| <i>l</i> -Asparagine hydrate..... | 8350 | 8350 | 8000 \pm 100 |
| <i>l</i> -Asparagine anhydrous..... | | | 5750 \pm 100 |
| <i>l</i> -Aspartic acid..... | 6190 | 5580 | 6000 \pm 100 |
| <i>dl</i> -Aspartic acid..... | 7200 | 6500 | 7100 \pm 100 |
| | 7100 (289°) | 6400 (289°) | 7250 \pm 70 (289°) |
| <i>l</i> -Cystine..... | 5500 | 5500 | |
| Diiodo- <i>l</i> -tyrosine..... | 7830 | 7830 | |
| <i>d</i> -Glutamic acid..... | 6550 | 6050 | 6330 \pm 75 |
| <i>dl</i> -Glutamic acid..... | 6180 | 5710 | |
| Glycine..... | 3590 | 3370 | 3355 \pm 15 |
| | 3660 (289°) | 3430 (289°) | 3580 \pm 40 (289°) |
| <i>l</i> -Histidine..... | | | 3200 \pm 100 (for 0.5 mole) |
| <i>l</i> -Hydroxyproline..... | | | > 1500 \pm 75 (for 2.0 mole) |
| <i>d</i> -Isoleucine..... | 843 | 843 | |
| <i>dl</i> -Isoleucine..... | 1790 | 1790 | |
| <i>l</i> -Leucine..... | 770 | 830 | |
| <i>dl</i> -Leucine..... | 2000 | 2070 | |
| <i>d</i> -Lysine..... | | | < - 3000 \pm 100 (for 1.0 mole) |
| <i>dl</i> -Methionine..... | 4240 | 4240 | 4100 \pm 100 |
| <i>dl</i> -Norleucine..... | 2540 | 2540 | |
| <i>l</i> -Phenylalanine..... | 2830 | 2830 | |
| <i>dl</i> -Phenylalanine..... | 2770 | 2770 | |
| <i>l</i> -Proline..... | | | > 300 \pm 50 (for 8.0 mole) |
| <i>dl</i> -Serine..... | 5414 | 5414 | 5050 \pm 60 |
| Taurine..... | 5950 | 5950 | 5700 \pm 100 |
| <i>l</i> -Tryptophan..... | 1360 | 1360 | |
| <i>l</i> -Tyrosine..... | 5960 | 5960 | |
| <i>dl</i> -Valine..... | 1500 | 1590 | 1730 \pm 25 |

* Dalton, J. B., and Schmidt, C. L. A.: *J. Biol. Chem.*, 103, 549 (1933).

† Corrected for the activity of the amino acid when possible.

The heat capacity of the solvent is determined by plotting n_2/n_1 against \bar{C}_{p_2} and integrating graphically by counting squares. The data are given in Table 15.

The partial molal heat capacity of amino acids in the standard solution is calculated with the aid of the equation

$$d \Delta H/dT = \Delta C_p = C_p (\text{products}) - C_p (\text{reactants})$$

The integral heat of dilution is given by

$$n_2(\bar{H}_2 - \bar{H}_2^\circ) + n_1(\bar{H}_1 - \bar{H}_1^\circ)$$

TABLE 15*
THE SPECIFIC HEAT AND HEAT CAPACITY OF AQUEOUS SOLUTIONS OF GLYCINE, *d,l*-ALANINE AND *d,l*-VALINE AT 25°

| m | GLYCINE. | | | | <i>d,l</i> -ALANINE. | | | | <i>d,l</i> -VALINE. | | | |
|------|----------------------|----------------------|-------------|----------------------|----------------------|----------------------|-------------|----------------------|----------------------|----------------------|-------------|----------------------|
| | Specific heat. | Heat capacity. | | | Specific heat. | Heat capacity. | | | Specific heat. | Heat capacity. | | |
| | | \overline{C}_{p_2} | φ_c | \overline{C}_{p_1} | | \overline{C}_{p_2} | φ_c | \overline{C}_{p_1} | | \overline{C}_{p_2} | φ_c | \overline{C}_{p_1} |
| | <i>cal. gm. deg.</i> | <i>cal.</i> | <i>cal.</i> | <i>cal.</i> | <i>cal. gm. deg.</i> | <i>cal.</i> | <i>cal.</i> | <i>cal.</i> | <i>cal. gm. deg.</i> | <i>cal.</i> | <i>cal.</i> | <i>cal.</i> |
| 0 | 0.998 | 7.5 | 7.5 | 17.98 | 0.998 | 40.0 | 40.0 | 17.98 | 0.998 | 93 | 93 | 17.98 |
| 0.2 | 0.985 | 8.5 | 7.5 | 17.98 | 0.988 | 39.0 | 39.5 | 17.98 | 0.993 | 84 | 90 | 18.01 |
| 0.5 | 0.965 | 9.0 | 8.0 | 17.97 | 0.974 | 37.0 | 38.0 | 17.99 | 0.983 | 69 | 85 | 18.09 |
| 0.63 | | | | | | | | | 0.976 | 63 | 82 | 18.15 |
| 1.0 | 0.936 | 11.5 | 8.5 | 17.94 | 0.950 | 34.0 | 37.0 | 18.04 | | | | |
| 1.5 | 0.910 | 14.0 | 9.5 | 17.90 | 0.928 | 31.0 | 35.5 | 18.13 | | | | |
| 1.88 | | | | | 0.910 | 29.0 | 35.0 | 18.16 | | | | |
| 2.0 | 0.886 | 16.5 | 11.0 | 17.84 | | | | | | | | |
| 2.5 | 0.867 | 19.5 | 13.0 | 17.73 | | | | | | | | |
| 3.0 | 0.851 | 22.0 | 15.0 | 17.66 | | | | | | | | |
| 3.33 | 0.840 | 23.5 | 15.5 | 17.56 | | | | | | | | |

* Unpublished data of Zittle, C. A., and Schmidt, C. L. A., to appear in *J. Biol. Chem.*, Jan., 1935.

TABLE 16*
THE RELATIVE APPARENT MOLAL HEAT CONTENT ($\varphi_h - \varphi_h^\circ$), IN CALORIES, OF CERTAIN AMINO ACIDS IN AQUEOUS SOLUTION AT 25°

| m | d-Arginine. | d-Lysine. | L-Histidine. | d-Pyrroglutamic acid. | L-Proline. | L-Hydroxyproline. | d,L-Serine. | Taurine. |
|-----|-------------|-----------|--------------|-----------------------|------------|-------------------|-------------|-----------|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | -165 | 135 | -60 ± 25 | -37 | 45 | 0 | -75 ± 25 | -105 |
| 1.0 | -240 ± 15† | 255 ± 15 | -105 | -75 | 97 | 0 | | -157 ± 18 |
| 1.5 | -300 | 420 | | -120 | 150 | -5 | | |
| 2.0 | | | | -180 | 195 | -10 | | |
| 3.0 | | | | -240 | 270 | -15 | | |
| 4.0 | | | | -315 | 345 | -22 ± 5 | | |
| 5.0 | | | | -390 | 405 | | | |
| 6.0 | | | | -480 | 465 | | | |
| 7.0 | | | | -540 ± 3.5 | 510 | | | |
| 8.0 | | | | | 570 ± 2.5 | | | |

* Unpublished data of Zittle, C. A., and Schmidt, C. L. A., to appear in *J. Biol. Chem.*, Jan., 1935.

† See footnote in Table XVIII.

where $(\bar{H} - \bar{H}^\circ) =$ total relative heat content, and $n =$ number of moles. These data are given in Table 16.

The integral heats of dilution of glycine calculated from the heat content of the constituents, together with those obtained by direct measurement, are given in Table 17.

TABLE 17*
HEAT OF DILUTION OF GLYCINE

| m. | Measured directly cal. per mol. | Calculated cal. per mol. |
|------|------------------------------------|-----------------------------|
| 0.02 | 0 | — |
| 0.04 | 2 | — |
| 0.10 | 12 | 25 |
| 0.33 | 45 | 52 |
| 0.50 | 70 | 68 |
| 1.00 | 125 | 126 |
| 3.00 | 210 | 235 |
| 3.33 | 225 | 250 |

* Unpublished data of Zittle, C. A., and Schmidt, C. L. A.: To appear in *J. Biol. Chem.*, Jan., 1935.

The relative partial molal heat contents of solvent and of solute have also been determined by Zittle and Schmidt. The heat contents of solute and solvent are given by the equations

$$(\bar{H}_2 - \bar{H}_2^\circ) = (\varphi_h - \varphi_h^\circ) + m \cdot d(\varphi_h - \varphi_h^\circ)/dm$$

$$(\bar{H}_1 - \bar{H}_1^\circ) = -m^2/55.5 \cdot d(\varphi_h - \varphi_h^\circ)/dm$$

By plotting the relative apparent molal heat contents of the solute $(\phi - \phi_h^\circ)$, obtained by measurement, against the molality, the slope required for the calculation may be obtained. The values for the relative apparent molal heat contents (integral heats of dilution) are given in Table 18.

Miyamoto and Schmidt have determined the free energy change, ΔF , of the amino acids due to ionization. These values, together with values for ΔH , the heat of ionization, and ΔS , the entropy change of the system due to ionization, are given in Table 1.* The calculations for ΔH are based on the relation

$$-\Delta H = \frac{d(R \ln K)}{d\left(\frac{1}{T}\right)} = \frac{4.5787 d \log K}{d\left(\frac{1}{T}\right)}$$

* For convenience, the term, ΔF , etc., is used in the text instead of the apparent values $(\Delta F')$, etc.

TABLE 18*

 THE RELATIVE PARTIAL MOLAL HEAT CONTENT, IN CALORIES, OF THE CONSTITUENTS OF AQUEOUS SOLUTIONS OF GLYCINE, *d,l*-ALANINE AND *d,l*-VALINE AT 25°

| m | GLYCINE. | | | | <i>d,l</i> -ALANINE. | | | | <i>d,l</i> -VALINE. | | | |
|------|-------------------------------|-------------------------------|-------------------------------|--|-------------------------------|-------------------------------|-------------------------------|--|-------------------------------|-------------------------------|-------------------------------|--|
| | $\bar{H}_2 - \bar{H}_2^\circ$ | $\varphi_h - \varphi_h^\circ$ | $\bar{H}_1 - \bar{H}_1^\circ$ | | $\bar{H}_2 - \bar{H}_2^\circ$ | $\varphi_h - \varphi_h^\circ$ | $\bar{H}_1 - \bar{H}_1^\circ$ | | $\bar{H}_2 - \bar{H}_2^\circ$ | $\varphi_h - \varphi_h^\circ$ | $\bar{H}_1 - \bar{H}_1^\circ$ | |
| 0 | 0 | 0 | 0 | | 0 | 0 | 0 | | 0 | 0 | 0 | |
| 0.1 | -25 | -12 | 0 | | 10 | 10 | 0 | | 40 | 15 | 0 | |
| 0.2 | -60 | -30 | 0.1 | | 30 | 15 | -0.1 | | 100 | 60 | -0.3 | |
| 0.5 | -135 | -70 | 0.6 | | 65 | 30 | -0.3 | | 245 | 125 | -1.1 | |
| 0.63 | | | | | | | | | 300 | 150 ± 25 | -1.5 ± 0.2 | |
| 1.0 | -215 | -125 | 1.6 | | 110 | 55 | -1.0 | | | | | |
| 1.5 | -280 | -155 | 2.9 | | 165 | 75 | -2.4 | | | | | |
| 1.88 | | | | | 210 | 100 ± 10 | -3.7 ± 0.2 | | | | | |
| 2.0 | -325 | -180 | 4.6 | | | | | | | | | |
| 2.5 | -360 | -195 | 6.3 | | | | | | | | | |
| 3.0 | -380 | -215 | 7.6 | | | | | | | | | |
| 3.33 | -400 | -230 ± 5† | 9.0 ± 0.2 | | | | | | | | | |

 * Unpublished data of Zittle, C. A., and Schmidt, C. L. A., to appear in *J. Biol. Chem.*, Jan., 1935.

† The uncertainty is indicated for the most concentrated solution. In more dilute solutions, where the heat effects are smaller, the percentage of uncertainty is greater.

where K = the true dissociation constant. The assumption was made that the equation holds when values for the apparent dissociation constants of the amino acids are used instead of the true dissociation constants (to calculate the latter values the activity coefficients are necessary) and that the increment, ΔH , is constant over the temperature range of 0° – 25° C. Values for the apparent dissociation constants at 0° and 25° C. were used in the calculation. Values for ΔF^{**} were calculated with the aid of the equation

$$(\Delta F^{298}) = -RT \ln K$$

The relation between (ΔS) , (ΔF) and (ΔH) is given by

$$(\Delta F) - (\Delta H) = -T(\Delta S)$$

The relationship between C_p and the entropy, S , is given by the equation

$$C_p = \left(\frac{\delta S}{\delta T} \right)_p$$

Thermal data for a number of the amino acids have been obtained by Borsook and Huffman, and by Parks and his coworkers. They measured the specific heats of the amino acids given over the temperature range from 90° K. to 298.1° K. These data permitted them

TABLE 19*
MOLAL ENTROPIES

| Substance. | S_{90° . | $\Delta S_{90-298.1^\circ}$. | $S_{298.1^\circ}$. |
|-----------------------------------|------------------|-------------------------------|---------------------|
| <i>d</i> -Alanine..... | 8.88 | 22.77 | 31.6 |
| <i>l</i> -Asparagine..... | 11.90 | 29.84 | 41.7 |
| <i>l</i> -Asparagine hydrate..... | 13.57 | 37.46 | 51.0 |
| <i>l</i> -Aspartic acid..... | 12.21 | 29.28 | 41.5 |
| <i>d</i> -Glutamic acid..... | 13.00 | 32.73 | 45.7 |
| Glycine..... | 7.55 | 18.57 | 26.1 |

S_{90° = entropy increment 0 – 90° K.

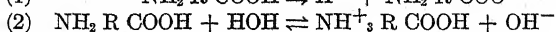
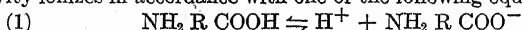
$\Delta S_{90-298.1^\circ}$ = entropy change between 90 – 298.1° K.

$S_{298.1^\circ}$ = entropy at 298.1° K.

* Huffman, H. M., and Borsook, H.: *J. Am. Chem. Soc.*, 54, 4297 (1932).

to calculate the molal entropies at 298.1° K. using the extrapolation method of Kelley, Parks and Huffman to calculate the entropy at 90° K. For the entropy change between 90° and 298.1° K., graphical integration was employed. These data are summarized in Table 19.

* ΔF° is defined for the present purposes as the free energy change when from an infinite volume of solution at 25° and a pressure of 1 atmosphere an amino acid at unit activity ionizes in accordance with one of the following equations:



yielding ions which are also at unit activity.

Using the heats of combustion data given in International Critical Tables (Vol. V, p. 167), the heats of formation were computed. By use of the relation,

$$(\Delta F) = (\Delta H) - T(\Delta S)$$

it was possible to calculate the free energies of formation. The data are given in Table 20.

TABLE 20*
THERMAL DATA AT 298.1° K

(The 15 cal. is used throughout and all weights are reduced to vacuum.)

| Substance. | Heat of combustion at constant pressure, cal. | ΔH_{298} , cal. | ΔS_{298} , E. U. | ΔF_{298} , cal. |
|-----------------------------------|---|-------------------------|--------------------------|-------------------------|
| <i>d</i> -Alanine..... | 387,200 | -134,600 | -153.5 | - 88,800 |
| <i>l</i> -Asparagine..... | 463,100 | -187,100 | -207.7 | -125,200 |
| <i>l</i> -Asparagine hydrate..... | 459,400 | -259,100 | -254.2 | -183,300 |
| <i>l</i> -Aspartic acid..... | 384,700 | -231,300 | -193.9 | -173,500 |
| <i>d</i> -Glutamic acid..... | 542,200 | -236,400 | -222.3 | -170,200 |
| Glycine..... | 233,800 | -125,460 | -126.5 | - 87,800 |

* Huffman, H. M., and Borsook, H.: *J. Am. Chem. Soc.*, **54**, 4297 (1932).

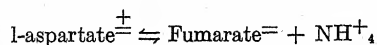
Borsook and Huffman have also determined the free energies of formation of aqueous solutions of certain of the amino acids by use of the above data, solubility data, the available activity data, and the free energies of dissociation as calculated from dissociation constants. Their data are given in Table 21. The free energy of deamination

TABLE 21*
FREE ENERGIES (CALORIES) OF FORMATION OF:

| | Crystalline. | Neutral 1 M. | Monovalent cation 1 M. | Monovalent anion 1 M. | Divalent anion 1 M. |
|------------------------------|--------------|--------------|------------------------|-----------------------|---------------------|
| <i>d</i> -Alanine..... | - 88,850 | - 89,200 | - 92,400 | - 76,000 | |
| <i>l</i> -Aspartic acid..... | -173,500 | -171,300 | -173,900 | -166,400 | -153,450 |
| <i>d</i> -Glutamic acid..... | -170,200 | -168,400 | -171,250 | -162,850 | -149,900 |

* Borsook, H., and Huffman, H. M.: *J. Biol. Chem.*, **99**, 663 (1933).

of aspartic acid was also calculated from the heat data and from the data on the equilibrium constant of the reaction,



which was obtained by Woolf (*Biochem. J.*, **23**, 472 (1929)) when toluene treated *B. coli communis* were added to the system. The equi-

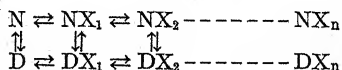
librium constant at 37° C. has the approximate value of 0.01. The free energy values obtained are as follows:

| | <i>Calories.</i> |
|---|------------------|
| $-\Delta F$ (from thermal data) | -4,750 |
| | -2,950 |
| | -2,250 |
| $-\Delta F$ (equilibrium constant, uncorrected for activity)... | -2,800 |
| $-\Delta F$ (equilibrium constant corrected for activity) | -3,700 |

DENATURATION

Denaturation of proteins involves a change in solubility resulting from treatment with acid or alkali and such substances as urea and sodium salicylate. It may also be brought about by subjecting the protein to heat, light or pressure. A characteristic of the phenomenon is that the temperature coefficient, about 75,000 calories, is high. However, this does not hold for acid denaturation. No single fact characterizes the various types of denaturation; hence, it is best in speaking of denaturation, to also state the type. The chemical changes involved are not known. There is an increase in the number of $-\text{SH}$ groups. Whether this is fundamental to denaturation or only incidental has not yet been established.

Anson and Mirsky have shown that denaturation is reversible. Hemoglobin, denatured by salicylate, has three properties characteristic of hemoglobin denatured by other means, *viz.*, (a) it is attacked by trypsin which does not digest native hemoglobin, (b) it is insoluble under the same conditions under which native hemoglobin is soluble, and (c) it possesses the parahematin type of spectrum which is also given by hemin when dissolved in pyridine. When denaturation of hemoglobin by salicylate is reversed, the original properties of hemoglobin are restored. When the concentration of salicylate is plotted against percentage of denaturation, a sigmoid curve is obtained. In the case of trypsin, temperature has but little effect on the equilibrium between the native and denatured product. Anson and Mirsky have advanced the idea that denaturation is caused by the addition of any substance which possesses a greater affinity for the denatured than for the native form. When a substance, X, is added to a protein solution, a series of compounds with X are formed which may be represented by



The sigmoid curve may be obtained by substituting a suitable set of values in the complex equilibrium equation. However, until the various equilibria concerned, together with the values for the constants, are known, an adequate explanation of the mechanism of denaturation will be lacking. According to Loughlin and Lewis, denaturation consists in a change from the molecular to the micellar state.

DIELECTRIC CONSTANTS AND SOLUBILITY RELATIONS

In recent years, Wyman and his coworkers have carried out extensive measurements on the dielectric constants of amino-acid solutions. Some of the data are given in Table 22.

TABLE 22*

DIELECTRIC CONSTANTS OF AMINO ACIDS AND PEPTIDS ACCORDING TO WYMAN

| Solute. | Solvent. | Concn. range. | ϵ |
|----------------------------------|--|---------------|--------------------------|
| Glycine..... | Water | 0-2.5 | 78.54 + 22.58 C |
| Glycine..... | 20 per cent Ethyl alcohol | 0-1.27 | 69.96 + 22.55 C |
| Glycine..... | 40 per cent Ethyl alcohol | 0-0.470 | 59.69 + 21.7 C |
| Glycine..... | 60 per cent Ethyl alcohol | 0-0.133 | 47.88 + 20.4 C |
| Glycine..... | 2.5 Molar urea | 0-2.17 | 84.35 + 22.3 C |
| Glycine..... | 5.0 Molar urea | 0-2.45 | 90.60 + 22.6 C |
| Glycine..... | 0.5898 Molar α -aminobutyric acid | 0-2.40 | 92.30 + 21.9 C |
| Glycine..... | 1.198 Molar α -aminobutyric acid | 0-2.30 | 106.75 + 21.0 C |
| Glycine..... | 1.826 Molar α -aminobutyric acid | 0-2.20 | 121.5 + 20.6 C |
| α -Aminobutyric acid..... | Water | 0-2.04 | 78.54 + 23.53 C |
| α -Aminobutyric acid..... | 20 per cent Ethyl alcohol | 0-0.9776 | 69.82 + 23.6 C |
| α -Aminobutyric acid..... | 40 per cent Ethyl alcohol | 0-0.5730 | 59.41 + 22.6 C |
| α -Aminobutyric acid..... | 60 per cent Ethyl alcohol | 0-0.2932 | 47.20 + 22.1 C |
| α -Aminobutyric acid..... | 80 per cent Ethyl alcohol | 0-0.0644 | 35.37 + 24.0 C |
| α -Aminobutyric acid..... | 2.793 Molar urea | 0-1.975 | 87.37 + 20.9 C |
| α -Aminobutyric acid..... | 0.961 Molar glycine | 0-0.7217 | 99.8 + 23.1 C |
| α -Aminobutyric acid..... | 1.993 Molar glycine | 0-1.27 | 124.05 + 19.0 C |
| α -Aminobutyric acid..... | 2.510 Molar glycine | 0-1.29 | 134.9 + 18.4 C |
| Glycine hexapeptide..... | Water | 0-0.0132 | 78.54 + 234 C |
| Glycine hexapeptide..... | 2.582 Molar urea | 0-0.0179 | 85.35 + 221 C |
| Glycine heptapeptide..... | 5.14 Molar urea | 0-0.00313 | 91.36 + (290 \pm 25) C |

ϵ = Linear expression of the dielectric constant, the first term of which is the dielectric constant of the solvent, while the coefficient of the concentrate C (moles per liter) in the second term is the slope of the curve.

* Wyman, Jr., *J. Am. Chem. Soc.*, 56, 536 (1934).

The dielectric constant has a pronounced influence in determining solubility of amino acids and proteins in a particular solvent. Other factors are: (a) Temperature, (b) nature of the solvent, (c) nature of the molecule, which includes such factors as molecular weight, free groups, and length of the hydrocarbon chain, and (d) dipole moment. Some amino acids and proteins are more soluble in water than in alcohol or in alcohol-water mixtures. Glycine is more soluble in water than α -alanine, although the dielectric constants of the two amino acids are essentially the same. β -Alanine, with a greater dipole moment, is much more soluble in water than α -alanine. The former amino acid increases the dielectric constant of water about 40 per

mole, while the corresponding increase for α -alanine is about 22. Amino acids with the shorter chains are more soluble than the longer chain amino acids. In alcohol-water mixtures, the amino acids with the longer chains are the more soluble. Proline is quite soluble in water and in alcohol. The prolamines are more soluble in alcohol-water mixtures than in water or in alcohol. The influence on solubility of inorganic salts varies with the nature of the salt and the amino acid. In general, amino acids whose solubilities are decreased most by alcohol are more soluble in salt solutions than in water.

Highly polar molecules are most soluble in highly polar solvents, while slightly polar or nonpolar molecules are soluble in nonpolar solvents or solvents of a low degree of polarity. Now water molecules are themselves dipoles and possess a high dielectric constant. Due to their zwitterion structure, amino acids and proteins are also dipoles.* As already stated, they contribute to the dielectric constant of the solution. Substances such as urea and inorganic salts which increase the dielectric constant also increase the solubility of the amino acid. Alcohol which lowers the dielectric constant when added to water decreases the solubility of amino acids and proteins. Amino acids whose dipoles are further separated than in an amino acid such as glycine are very soluble and are less influenced by the addition of neutral salts, while the slightly soluble amino acids, such as glutamic acid and cystine, which contribute very little to the dielectric constant of the environment, are rendered very much less soluble when neutral salts are added. A summary of the subject is given by Cohn.

Wyman and others have shown in the case of the amino acids and peptides which were studied that the dielectric constant, within the limits studied, was a linear function of the concentration, or,

$$\delta = \frac{\epsilon - \epsilon_0}{C}$$

where δ = increase in dielectric constant per mole, ϵ = dielectric constant of the solution, ϵ_0 = dielectric constant of water, and C = concentration of ampholyte in moles per liter. The effect of increasing the size of the amino-acid molecule has no marked influence on the dielectric constant. For a possible interpretation of these findings, the reader is referred to the papers of Wyman.

The solubility relationships of proteins resemble in many ways those of the amino acids. The solubility is determined by the chemical makeup and size of the protein molecule and the nature and concentration of the neutral salts. When the concentration of neutral salt is increased sufficiently, salting-out occurs. The relations can be expressed by the equation,

$$\log S = \beta - K_s/\mu$$

* This is only true for the monoamino-monocarboxylic acids and for the zwitterion portions of the hexone bases, the dicarboxylic amino acids, and the proteins possessing excess basic or acidic groups.

where S = solubility, μ = the ionic strength, β = an intercept constant, and K_s = salting-out constant. The relations have been experimentally verified by Florkin's studies on fibrin and Green's work on hemoglobin. At pH 6.6 and 25° C., Green found that the solubility of horse carboxyhemoglobin in various chloride and sulfate solutions can be expressed by the equation,

$$\log S = 1.30 + 1.6 \sqrt{C} - k_0 C$$

where k_0 is an empirical constant varying with the electrolyte. The activity coefficient of hemoglobin in these salt solutions can be approximately described in terms of a simplified Debye-Hückel equation,

$$-\log \gamma = \log S - \log S_0 = \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + A} - K_s$$

where $\log S_0 = 1.20$, $Z_1 Z_2$ is 4, and A and K_s are empirical constants.

(The reader is referred to the articles in the *Annual Review of Biochemistry* for summaries of the recent progress in the field of amino acids and proteins.)

CARL L. A. SCHMIDT.

REFERENCES

General and Reviews

1. Abramson, H. A.: *Electrokinetic Phenomena and Their Application to Biology and Medicine* (1934).
2. Cohn, E. J.: *Physiol. Rev.*, **5**, 349 (1925); *Ergebnisse Physiol.*, **33**, 781 (1931).
3. Loeb, J.: *Proteins and the Theory of Colloidal Behavior* (1922).
4. Michaelis, L.: *Hydrogen Ion Concentration*. Trans. by Perlzweig, Vol. I (1926); *Die allgemeine Bedeutung der Wasserstoffionkonzentration für die Biologie*, in Oppenheimer, C.: *Handbuch der Biochemie des Menschen und der Tiere* (1913), *Ergänzungsband*, p. 10.
5. Pauli, W., and Valkó, E.: *Electrochemie der Kolloide* (1929); *Kolloidchemie der Eiweisskörper* (Dresden, 1933).
6. Tillmans, J., and Hirsch, P.: *Handbuch der Lebensmittelchemie*, Vol. I; 117 (1933).

Titration Curves and Dissociation Constants of Amino Acids

7. Dalton, J. B., Kirk, P. L., and Schmidt, C. L. A.: *J. Biol. Chem.*, **88**, 589 (1930).
8. Emerson, O. H., Kirk, P. L., and Schmidt, C. L. A.: *J. Biol. Chem.*, **92**, 449 (1931).
9. Foster, G. L., and Schmidt, C. L. A.: *J. Biol. Chem.*, **56**, 545 (1923); *J. Amer. Chem. Soc.*, **48**, 1709 (1926).
10. Hitchcock, D. I.: *J. Gen. Physiol.*, **6**, 747 (1923-24).
11. Kirk, P. L., and Schmidt, C. L. A.: *Univ. of Calif. Pub. Physiol.*, **7**, 57 (1929); *J. Biol. Chem.*, **81**, 237 (1929).
12. Margaria, R., and Green, A. A.: *J. Biol. Chem.*, **102**, 611 (1933).
13. Merrill, A. R. T.: *J. Am. Chem. Soc.*, **43**, 2688 (1921).
14. Miyamoto, S., and Schmidt, C. L. A.: *J. Biol. Chem.*, **87**, 327 (1930); **90**, 165 (1931).
15. Nims, L. F., and Smith, P. K.: *J. Biol. Chem.*, **101**, 401 (1933).
16. Owen, B. B.: *J. Am. Chem. Soc.*, **56**, 24 (1934).
17. Sano, K.: *Biochem. Z.*, **168**, 14 (1926).
18. Schmidt, C. L. A., Appleman, W. K., and Kirk, P. L.: *J. Biol. Chem.*, **81**, 723 (1929).
19. Schmidt, C. L. A., Kirk, P. L., and Appleman, W. K.: *J. Biol. Chem.*, **88**, 285 (1930).
20. Simms, H. S.: *J. Am. Chem. Soc.*, **48**, 1239 and 1251 (1926).

21. Winkelblech, K.: *Z. physik. Chem.*, **36**, 546 (1901).
 22. Wood, J. K.: *J. Chem. Soc.*, **105**, 1988 (1914).

(With Formaldehyde)

23. Harris, L. J.: *Proc. Roy. Soc. (London)*, **104B**, 412 (1929).
 24. Levy, M.: *J. Biol. Chem.*, **99**, 767 (1933).

(In Alcohol)

25. Jukes, T. H., and Schmidt, C. L. A.: *J. Biol. Chem.*, **105**, 359 (1934).
 26. Neuberger, A.: *Proc. Roy. Soc. (London)*, **115B**, 180 (1934).

Zwitter-ions

27. Borsook, H., and MacFadyen, D. A.: *J. Gen. Physiol.*, **13**, 509 (1930).
 28. Edsall, J. T., and Blanchard, M. H.: *J. Am. Chem. Soc.*, **55**, 2337 (1933).
 29. Harris, L. J.: *Biochem. J.*, **24**, 1080 (1930).
 30. Miyamoto, S., and Schmidt, C. L. A.: *Univ. of Calif. Pub. Physiol.*, **8**, 1 (1932).
 31. Richardson, G. M.: *Proc. Roy. Soc. (London)*, **115B**, 121 (1934).
 32. Scatchard, G., and Kirkwood, J. G.: *Physik. Z.*, **33**, 22 and 297 (1932).

Activity Coefficients of Amino Acids and Proteins

33. Adair, G. S.: *J. Am. Chem. Soc.*, **51**, 696 (1929).
 34. Hoskins, W. M., Randall, M., and Schmidt, C. L. A.: *J. Biol. Chem.*, **88**, 215 (1930).
 35. Dalton, J. B., and Schmidt, C. L. A.: *J. Biol. Chem.*, **103**, 549 (1933).

Combination of Proteins with Acids and Bases

36. Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A.: *J. Biol. Chem.* **72**, 707 (1927).
 37. Czarnetzky, E. J., and Schmidt, C. L. A.: *J. Biol. Chem.*, **105**, 301 (1934).
 38. Hitchcock, D. I.: *J. Gen. Physiol.*, **16**, 357 (1932).
 39. Kirk, P. L., and Schmidt, C. L. A.: *J. Biol. Chem.*, **76**, 115 (1928).
 40. Rawlins, L. M. C., and Schmidt, C. L. A.: *J. Biol. Chem.*, **82**, 709 (1929); **88**, 271 (1930).

Compounds of Proteins and Amino Acids with Heavy Metals

41. Borsook, H., and Thimann, K. V.: *J. Biol. Chem.*, **98**, 671 (1932).
 42. Ettisch, G., and Schulz, G. V.: *Biochem. Z.*, **245**, 189 (1932).
 43. Northrup, J. H., and Kunitz, M.: *J. Gen. Physiol.*, **5**, 481 (1928).
 44. Pauli, W., and Matula, J.: *Biochem. Z.*, **80**, 187 (1917).
 45. Smythe, C. V., and Schmidt, C. L. A.: *J. Biol. Chem.*, **88**, 241 (1930).
 46. Vickery, H. B., and Gordon, W. G.: *J. Biol. Chem.*, **103**, 543 (1933).

Transport

47. Greenberg, D. M.: *Univ. of Calif. Pub. Physiol.*, **7**, 9 (1927); *Trans. Am. Electrochem. Soc.*, **54**, 107 (1928).
 48. Greenberg, D. M., and Schmidt, C. L. A.: *J. Gen. Physiol.*, **7**, 287 and 317 (1924); **8**, 271 (1926).
 49. MacInnes, D. A., and Longsworth, L. G.: *Chem. Rev.*, **11**, 171 (1932).
 50. Miyamoto, S., and Schmidt, C. L. A.: *J. Biol. Chem.*, **99**, 335 (1933).

Molecular Weights

(a) Osmotic Pressure

51. Adair, G. S.: *Proc. Roy. Soc. (London)*, **109A**, 292 (1925); **120A**, 573 (1928); *J. Amer. Chem. Soc.*, **51**, 696 (1929); *Biochem. J.*, **24**, 1864 (1930).
 52. Burk, N. F.: *J. Biol. Chem.*, **98**, 353 (1932).
 53. Burk, N. F., and Greenberg, D. M.: *J. Biol. Chem.*, **87**, 197 (1930).
 54. Pauli, W., and Fent, P.: *Kolloid. Z.*, **67**, 288 (1934).

(b) Ultracentrifuge

55. Svedberg, T.: *J. Biol. Chem.*, **103**, 311 (1933); *Naturwissenschaften*, **22**, 225 (1934); *Chem. Rev.*, **14**, 1 (1934); *Kolloid. Z.*, **67**, 1 (1934).
 56. Svedberg, T., Carpenter, L. M., and Carpenter, D. C.: *J. Am. Chem. Soc.*, **52**, 241 (1930).

Conductivity

57. Gahl, R., Greenberg, D. M., and Schmidt, C. L. A.: *Univ. of Calif. Pub. Physiol.*, **5**, 289 and 307 (1926).
 58. Greenberg, D. M.: *J. Gen. Physiol.*, **7**, 303 (1924).
 59. Mehl, J. W., and Schmidt, C. L. A.: *J. Gen. Physiol.*, (in press).
 60. Miyamoto, S., and Schmidt, C. L. A.: *Univ. of Calif. Pub. Physiol.*, **8**, 9 (1932).

Solubility of Amino Acids

61. Cohn, E. J.: *Naturwissenschaften*, **20**, 663 (1932).
 62. Dalton, J. B., and Schmidt, C. L. A.: *J. Biol. Chem.*, **103**, 549 (1933).
 63. Dunn, M. S., Ross, F. J., and Read, L. S.: *J. Biol. Chem.*, **103**, 579 (1933).

Solubility of Proteins in Salt Solutions

64. Cohn, E. J.: *Naturwissenschaften*, **20**, 663 (1932).
 65. Florkin, M.: *J. Biol. Chem.*, **87**, 629 (1930).
 66. Green, A. A.: *J. Biol. Chem.*, **93**, 495 and 517 (1931).

Molar Volumes

67. Cohn, E. J., McMeekin, T. L., Edsall, J. T., and Blanchard, M. H.: *J. Am. Chem. Soc.*, **56**, 784 (1934).
 68. Dalton, J. B., and Schmidt, C. L. A.: *J. Biol. Chem.*, **103**, 549 (1933).
 69. Traube, J.: *Samml. Chem. u. Chem. tech. Vortr.*, **4**, 275 (1899).

Valence of Protein Ions

70. Adair, G. S., and Adair, M. E.: *Biochem. J.*, **28**, 199 (1934).

Diffusion of Proteins and Amino Acids

71. Longworth, L. G.: *J. Gen. Physiol.*, **17**, 211 (1933).
 72. McBain, J. W., and Dawson, C. R.: *J. Am. Chem. Soc.*, **56**, 52 (1934).
 73. Northrup, J. H., and Anson, M. L.: *J. Gen. Physiol.*, **12**, 543 (1929).
 74. Tiselius, A., and Gross, D.: *Kolloid. Z.*, **66**, 11 (1934).

Swelling

75. Greenberg, D. M., and Mackey, M. A.: *J. Gen. Physiol.*, **15**, 161 (1931).
 76. Northrup, J. H., and Kunitz, M.: *J. Phys. Chem.*, **35**, 162 (1931); *J. Gen. Physiol.*, **8**, 317, 893 and 905 (1926); **10**, 161 (1926).
 77. Wilson, J. H., and Wilson, W. H.: *J. Am. Chem. Soc.*, **40**, 886 (1918).

Optical Rotation

78. Almquist, H. J., and Greenberg, D. M.: *J. Biol. Chem.*, **93**, 167 (1931); **105**, 519 (1934).
 79. Lutz, O., and Jirgenson, B.: *Ber.*, **63**, 448 (1930); **64**, 221 (1931).

Ultrafiltration

80. Greenberg, D. M., and Greenberg, M.: *J. Biol. Chem.*, **94**, 373 (1931); *J. Gen. Physiol.*, **16**, 559 (1933).

Refractivity

81. Craig, R., and Schmidt, C. L. A.: *Australian J. Exp. Biol. Med. Sci.*, **9**, 33 (1932).

Denaturation

82. Anson, M. L., and Mirsky, A. E.: *J. Gen. Physiol.*, **14**, 605 (1931); **17**, 393 and 399 (1934); *J. Phys. Chem.*, **35**, 185 (1931).
83. Lewis, W. C. M.: *Chem. Rev.*, **8**, 81 (1931).
84. Mirsky, A. E., and Anson, M. L.: *J. Gen. Physiol.*, **13**, 121 and 133 (1929).
85. Rimington, C.: *Nature*, March 21 (1931).
86. Wu, H.: *Chinese J. Physiol.*, **5**, 321 (1931).

Dielectric Constants

87. Cohn, E. J., Thomas, L., McMeekin, T. L., and Edsall, J. T.: *Arch. sci. biol.*, **18**, No. 1 (1933).
87A. Debye, P., et al.: *Trans. Faraday Soc.*, **30**, 679 (1934).
88. Frankenthal, M.: *Z. physik. Chem.*, **21**, 310 (1933).
89. Heymann, E.: *Kolloid. Z.*, **66**, 229 (1934).
90. Loughlin, W. J., and Lewis, W. C. M.: *Biochem. J.*, **26**, 476 (1932).
91. Wyman, J.: *Phys. Rev.*, **35**, 623 (1930); *J. Biol. Chem.*, **90**, 443 (1931); *J. Am. Chem. Soc.*, **56**, 536 (1934).
92. Wyman, J., and McMeekin, T. L.: *J. Am. Chem. Soc.*, **55**, 908 and 915 (1933).

Thermodynamic Data

93. Adair, G. S., Cordero, N., and Shen, T. C.: *J. Physiol.*, **67**, 288 (1929).
94. Borsook, H., and Huffman, H. M.: *J. Biol. Chem.*, **99**, 663 (1933); *J. Amer. Chem. Soc.*, **54**, 4297 (1932).
95. Czarnetzky, E. J., and Schmidt, C. L. A.: *Z. physiol. Chem.*, **204**, 129 (1932); *J. Biol. Chem.*, **97**, 333 (1932).
96. Dalton, J. B., and Schmidt, C. L. A.: *J. Biol. Chem.*, **103**, 549 (1933).
97. Miyamoto, S., and Schmidt, C. L. A.: *J. Biol. Chem.*, **90**, 165 (1931).
98. Parks, G. S., Huffman, H. M., and Barmore, M.: *J. Am. Chem. Soc.*, **55**, 2733 (1933).
99. Zittle, C. A., and Schmidt, C. L. A.: *J. Biol. Chem.* (in press).

CHAPTER VII

THE NUCLEIC ACIDS

IN 1871 Miescher¹ announced the separation of the nuclear substance from the other substances of both pus cells and egg yolk. This material, which he termed *nuclein*, was isolated in the following manner: Pus cells were digested with dilute hydrochloric acid for several weeks. On shaking the product with ether, part of the solid material gathered at the interface between the ether and water, and a second solid layer (consisting of practically pure nuclein) settled at the bottom of the aqueous layer. The same material was obtained by digesting pus cells with artificial gastric juice, the undigestible residue being nuclein. Miescher's nuclein had stronger acidic properties than proteins were known to possess; it was insoluble in dilute acids but soluble in dilute alkalis. It was insoluble in water and in organic solvents. It contained carbon, hydrogen, nitrogen and phosphorus but no sulfur.

Using Miescher's method, Hoppe-Seyler² was able to isolate a nuclein from the yeast cell; Plosz studied the nuclear material from the red cells of birds and reptiles; and Lûbavin obtained a residue resembling Miescher's nuclein on digesting casein with artificial gastric juice. The general properties of these nucleins resembled those of Miescher's nuclein, but the elementary composition varied according to the source of the nuclein.

Miescher now turned his attention to the nuclear material from a readily accessible source—the ripe spermatozoa of the salmon. This nuclear material was found to consist of a salt of an organic acid with a basic substance which Miescher termed *protamine*.

The term "nucleic acid" was introduced by Altmann³ in 1889 to describe the acidic substance in combination with the protamine (a protein). Principally as a result of the work of Kossel who, in 1891, made the first hydrolysis of a protein-free nucleic acid, the nucleic acids were recognized as belonging to two groups, that from yeast being representative of one group (ribose nucleic acid), that from thymus gland and fish sperm being typical of the other group (desoxyribose nucleic acid). The products of hydrolysis of the two principal nucleic acids are shown in the following table:

Yeast nucleic acid

Adenine.
Guanine.
Cytosine.
Uracil.
d-Ribose.
Phosphoric acid.

Thymonucleic acid

Adenine.
Guanine.
Cytosine.
Thymine.
d-2-Desoxyribose.
Phosphoric acid.

Thus these two nucleic acids differ in composition with regard to the constituent sugar and one pyrimidine base. The striking difference in the chemical and physical properties of the two acids is due to the properties of the sugar component, so that the two classes are now known as the ribose nucleic acids and the desoxyribose nucleic acids, respectively. The earlier distinction between plant nucleic acid and animal nucleic acid has been abandoned with the definite discovery of ribose nucleic acid derivatives concomitantly with thymonucleic acid in animal tissues.

Preparation of Yeast Nucleic Acid.—The following is one of the simplest methods of isolating yeast nucleic acid:⁴ An aqueous solution of potassium hydroxide is added dropwise, with grinding, to a thick, aqueous paste of pressed yeast, until the mixture is faintly alkaline to litmus. The mixture is now thinned by the addition of saturated aqueous picric acid solution and filtered. On addition of hydrochloric acid to the yellow filtrate, nucleic acid separates in lumps, adhering to the bottom and walls of the containing vessel. The product, which is light yellow because of contamination with a little picric acid, is then purified. It is dissolved in a slight excess of dilute potassium hydroxide solution, filtered from a little insoluble material, and the filtrate rendered acid with acetic acid. The nucleic acid is precipitated from this solution by pouring into ten times its volume of ethyl alcohol.

The same material may be prepared from wheat embryo.

Preparation of Thymonucleic Acid.—The nucleic acid from thymus glands may be isolated in the following manner:⁵ The hashed thymus glands are mixed with 5 per cent aqueous sodium chloride and heated until boiling. A few cubic centimeters of acetic acid are then added and the boiling continued during five minutes. Sodium acetate and sodium hydroxide are now added and the mixture boiled a further five minutes, by which time most of the tissue has dissolved. The hot mixture is rendered neutral by the addition of glacial acid, colloidal iron and more glacial acetic acid are added and the mixture filtered while hot. The nucleic acid is precipitated from the filtrate by the addition of twice its volume of ethyl alcohol.

The product is purified by dissolving in the minimum quantity of dilute alkali and reprecipitating by the addition of dilute hydrochloric acid. The precipitate is filtered off and washed with alcohol until free from hydrochloric acid.

Substances of the type of thymonucleic acid have been isolated from the following sources: Fish sperm, thymus, spleen, pancreas, testicle, intestine, placenta, mammary glands, liver, brain, kidney, blood cells, bacteria, thyroid, lungs, mesenteric ganglia, lymphatic glands, lymphatic ganglia and tumor tissue.

THE NUCLEOSIDES

Certain free nucleosides occur in nature—for example, guanosine and vicine in plants, and inosine in beef extract. The others are found combined in the nucleotides and polynucleotides.

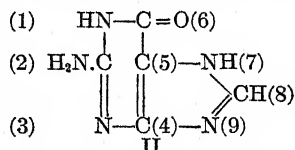
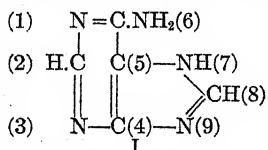
Ribose Nucleosides.—By hydrolysis of yeast nucleic acid with fairly dilute ammonia at 180° C. during three and one-half hours, the phosphoric acid is set free and four glycosides, termed *nucleosides*, are obtained—the ribosides of adenine, guanine, cytosine and uracil. These nucleosides are known as adenosine, guanosine, cytidine and uridine respectively. The discovery of this method of isolating the nucleosides obviously ended at once the dispute as to whether the bases in the molecule of ribose nucleic acid are attached to the sugar or to the phosphoric acid.

The ribose nucleosides are subdivided into two classes according to the nature of the base: (1) Purine ribosides; (2) pyrimidine ribosides.

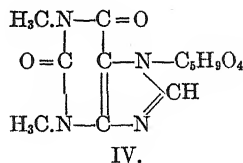
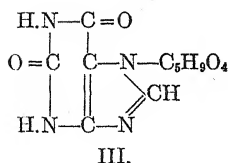
Purine Ribosides.—The two purine ribosides occurring in yeast nucleic acid are adenosine and guanosine. They are readily hydrolyzed by dilute mineral acid to give ribose and the base. The sugar is identified by its melting point and specific rotation and by the identity of its osazone and *p*-bromophenylosazone. In addition, it can be oxidized first to ribonic acid, identical with that prepared synthetically by Fischer and Piloty, and then to *i*-trihydroxyglutaric acid.

Owing to the ease of hydrolysis, the presence of a pentose is readily revealed by the orcinol test, and its amount may be determined by estimation of the furfural formed on treating with hydrochloric acid. On deamination, adenosine and guanosine give rise to inosine (hypoxanthine riboside) and xanthosine (xanthine riboside), respectively, which have the same sugar ring structure and point of union of sugar to base as have the parent nucleosides. Since they are all hydrolyzed by the same enzyme, the stereochemical disposition of the glycosidic union must be identical.

From a consideration of the formulae of adenine (I) and guanine (II), the position of union of ribose to the purine cannot be at positions

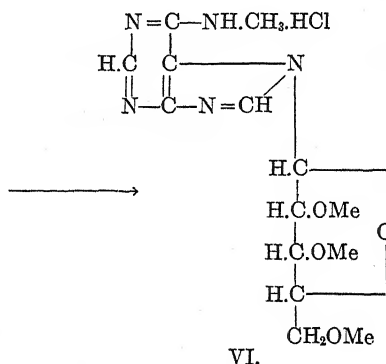
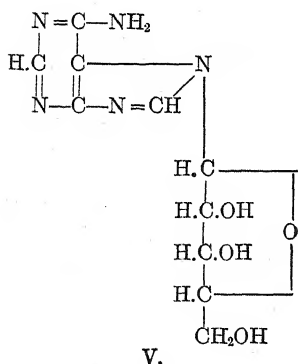


1 or 3 since there is no replaceable hydrogen; nor at position 2 because guanosine has a free amino group attached to that carbon atom; nor at position 6 since adenosine has a free amino group there. The union must therefore be at either position 7 or 8 of the purine. Now, by treating with diazomethane, Levene transformed xanthosine (III) into theophylline riboside (IV), showing definitely that position 7 was substituted. Had position 7 been unsubstituted, methylation would have proceeded in positions 1, 3 and 7 to give caffeine riboside.

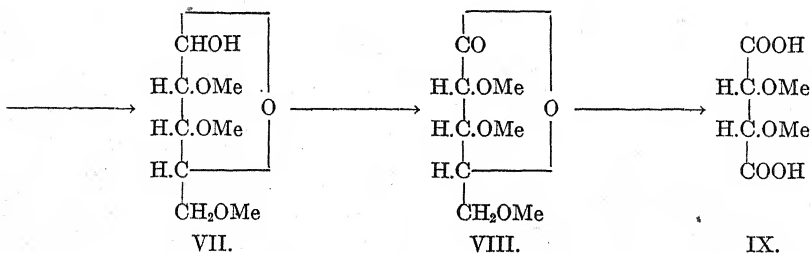


Hence adenosine, inosine, guanosine and xanthosine are all 7-ribosidopurines.

Turning now to the ring structure of the sugar component of the purine ribosides, Levene and Tipson⁶ prepared the completely methylated derivatives of adenosine (V) and guanosine by simultaneous de-



acetylation and methylation of the acetylated substances. In this way, trimethyl-N-methyl adenosine (VI) and trimethyl-N-methyl guanosine were formed and isolated as the hydrochlorides. On hydrolysis of the adenosine derivative by means of dilute hydrochloric acid, 6-methyl adenine and trimethyl ribofuranose (VII) were isolated. The same trimethyl sugar was isolated from the guanosine derivative and was identified in each case by oxidation first to trimethyl γ -ribonolac-

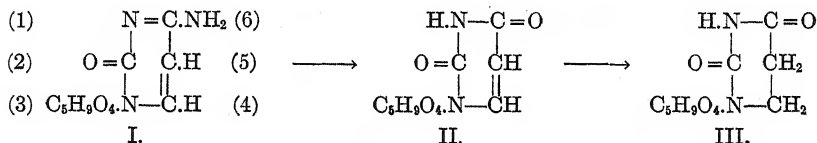


tone (VIII) and then to *i*-dimethoxysuccinic acid (IX). It follows that the sugar component has the furanose ring structure. This conclusion is confirmed by researches on the structure of inosinic acid (see ribonucleotides)

Pyrimidine Ribosides.—The two pyrimidine ribosides of yeast nucleic acid are cytidine and uridine. Since cytidine is readily transformed to uridine by deamination, the two substances have the same sugar ring structure and point of union of sugar to base.

The task of solving the problem of their structure has been beset with great difficulties. Unlike the purine ribosides, they cannot be hydrolyzed by dilute mineral acids; and when treated with more concentrated acid, the nitrogenous base is readily isolated but the sugar is destroyed. However, by simultaneous hydrolysis and oxidation of cytidine with hydrobromic acid and bromine, bromo-uracil and ribonic acid were obtained.

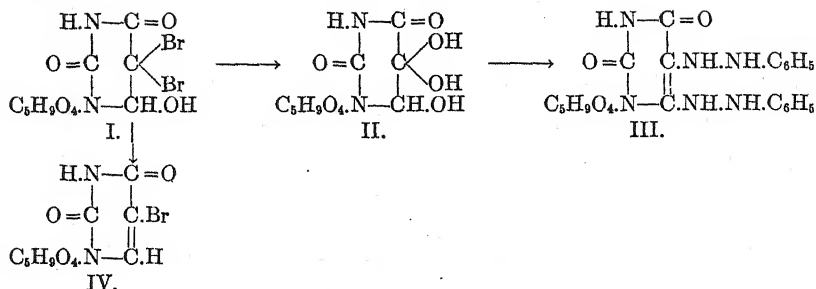
Though they give only a faint orcinol test, on prolonged distillation with hydrochloric acid the quantity of furfural formed is constant with the assumption of equimolecular proportions of base and ribose. On hydrogenation the characteristic stability is lost, and the dihydro derivative is capable of hydrolysis under conditions comparable with those used for the hydrolysis of the purine ribosides. Thus, hydrogenation of uridine (II) gives dihydro-uridine (III) which is readily hydrolyzed to dihydro-uracil and ribose.



Now, since there is a free primary amino group in cytidine at position 6, there can be no replaceable hydrogen atom at position 1, so that positions 1 and 6 are excluded as points of union of the base to the ribose both in cytidine (I) and in uridine (II), despite the presence of a replaceable hydrogen atom at position 1 in uridine. Direct experimental confirmation was provided by Levene and Tipson⁷ who treated diacetyl trityl uridine with diazomethane. On removal of the acetyl and trityl groups from the product, a monomethyl uridine was obtained. This was shown to be N(1)-methyl uridine, since on hydrolysis 1-methyl uracil was formed.

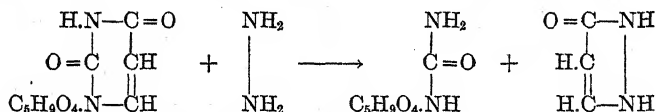
On treating uridine with nitric acid, 5-nitro-uridine carboxylic acid (which may be hydrolyzed to 5-nitro uracil) is formed. Likewise, bromine reacts with uridine to give 5-bromo-uridine (IV). Hence position 5 cannot be the point of union.

Furthermore, if bromine is added in slight excess to an aqueous solution of uridine, the excess bromine removed by aeration and the product treated with phenylhydrazine, diphenylhydrazino-uridine (III) results. This is explained by assuming the intermediate formation of 4-hydroxy-5,5-dibromodihydro-uridine (I) which then passes to a derivative of isodialuric acid (II).



This reaction is significant since it has been shown that unless position 3 in uracil is substituted and positions 4 and 5 are free, only the mono-phenylhydrazino-derivative is formed. Thus, 1,3-dimethyl uracil gives the di-, whereas uracil gives the monoderivative. It follows that in both uridine and cytidine the ribose residue is situated at position 3 of the base.

This conclusion is substantiated by the failure of uridine to give a dye with diazobenzene sulfonic acid or to give a purple color on applying the Wheeler and Johnson color test. These reactions do not take place with 3-substituted uracil derivatives. In addition, pyrazolone but no urea is formed by the action of hydrazine hydrate on uridine. Hence the union is at position 3 and not position 4, since in the latter case both a substituted pyrazolone and urea would have been isolated.



The furanose structure of the ribose portion of the molecule has been elucidated⁸ in the same general manner as for adenosine and guanosine.

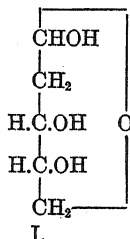
The Structure of the Desoxyribose Nucleosides.—The hydrolysis of thymonucleic acid to the constituent nucleosides is achieved by passing a solution of the acid through a segment of the gastro-intestinal tract of a dog and collecting it from an intestinal fistula.⁹ In order to avoid contamination with food remnants, a gastric fistula is created to establish a clean and empty segment.

A solution of 50 Gm. of thymonucleic acid is allowed to flow through the gastric fistula and the digestion mixture is collected from the intestinal fistula during one to two hours, the volume of fluid varying from 350 to 700 cc. The solution is then covered with a layer of toluene and incubated in a thermostat during four to seven days, small portions of gastro-intestinal secretions being added daily. The digestion mixture is now poured into twice its volume of 95 per cent alcohol, filtered and the nucleosides isolated from the filtrate.

As in the case of the ribose nucleosides, the desoxyribose nucleosides may be subdivided into two groups depending on the nature of the base: (1) purine desoxyribosides; (2) pyrimidine desoxyribosides.

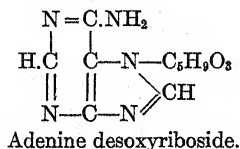
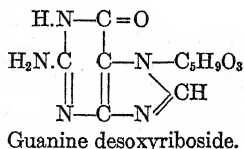
Purine Desoxyribosides.—The two purine desoxyribosides isolated from thymonucleic acid are glycosides of guanine and hypoxanthine. Identification of the nitrogenous base offered no difficulty and need not be discussed, but extreme difficulty was experienced in isolating and identifying the sugar component. The sugar was found to be much less stable than *d*-ribose or any other known sugar. In addition, it forms a very soluble hydrazone and no osazone, so that these means of isolating a derivative from the hydrolysis product were barred.

Levene^{9A} was able to isolate the crystalline sugar by extremely mild hydrolysis of very pure nucleoside. The nucleoside was warmed with 0.01 *N* mineral acid during ten minutes, a sugar being isolated having the formula $C_5H_{10}O_4$ (that is, having one atom of oxygen less than a pentose). Analysis of its benzylphenylhydrazone and of two of its purine and two pyrimidine nucleosides confirmed this composition. The sugar was identified as *d*-2-desoxyribose (I),



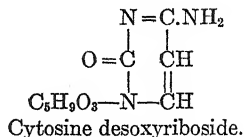
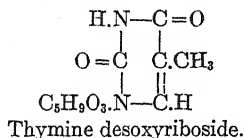
since synthetic *l*-2-desoxyribose, prepared by the action of dilute sulfuric acid on *l*-arabinal (*l*-ribal), had the same numerical values of initial and equilibrium specific rotation, the direction of rotation being opposite in sign. On treatment with dilute sulfuric acid, levulinic acid, $\text{CH}_3.\text{CO}.\text{CH}_2.\text{CH}_2.\text{COOH}$, was formed from both the natural and synthetic sugars. The natural sugar gives all the qualitative tests common to 2-desoxysugars.

The general arguments advanced to show the position of union of sugar to base in the purine ribosides hold also for the purine desoxyribosides. The structure of the two purine desoxyribosides may therefore be written as.



Pyrimidine Desoxyribosides.—The two pyrimidine desoxyribosides occurring in thymonucleic acid are glycosides of thymine and cytosine. On treatment with 5 per cent sulfuric acid, hydrolysis takes place, and the pyrimidine formed is readily recognizable, but the sugar liberated is immediately transformed to levulinic acid. By analogy with the

pyrimidine ribosides, position 3 of the base is assumed to be the point of glycosidic union with the sugar:



Two other important nucleosides, which are derivatives of neither ribose nor desoxyribose, may be mentioned.

Vicine is a nucleoside which has been isolated from vegetable sources (for example, vetch meal). On hydrolysis it gives divicine (4,6-dioxy-2,5-diaminopyrimidine) and *d*-glucose.

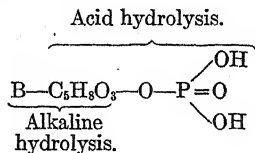
Adenine thiomethylpentoside is obtained from the alcoholic extract of brewers' yeast. It is readily hydrolyzed by mineral acids to give adenine and a thiomethylpentose.

NUCLEOTIDES

Certain free nucleotides (*e. g.*, guanylic and inosinic acids) occur in nature. The others are found in combination in the polynucleotides.

Purine Ribose Nucleotides.—By hydrolysis of yeast nucleic acid with very dilute ammonia at 115° C. for one hour, the phosphoric acid is not liberated, and the reaction product is an equimolecular mixture of the monophosphates of the four nucleosides. These substances, termed *nucleotides*, are known as adenylic, guanylic, cytidylic and uridylic acids respectively.

On alkaline hydrolysis of a nucleotide, the phosphoric acid is set free and the nucleoside is formed. On the other hand, by acid hydrolysis (of inosinic acid, for example) the phosphoric ester of ribose is formed. It is therefore obvious that in the nucleotides the phospho sugar is in glycosidic union with the base (B), so that their general formula may be represented as follows:



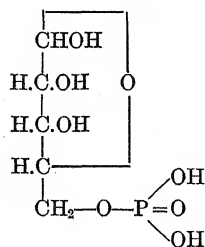
Since the structure of the ribonucleosides has already been discussed, we are now only concerned with the position of attachment of the phosphoric acid to the ribose residue. The purine ribose nucleotides have definitely been shown to belong to two classes. The first class consists of glycosides of 5-phosphoribose, derived from the ribose nucleic acid of animals; the second class are glycosides of 3-phosphoribose and may be prepared from yeast nucleic acid as indicated above.

Both the known 5-phosphoribosides are purine glycosides.

Muscle Inosinic Acid.—The first nucleotide to be discovered was named *inosinic acid* by Liebig¹⁰ who, in 1847, isolated it from beef extract. It was later found in the muscles of the chicken and the duck, and was also isolated from the herring.

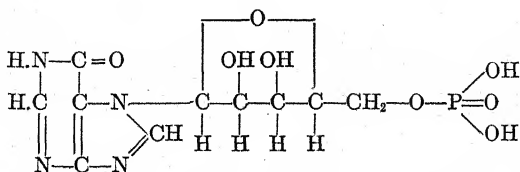
By boiling a solution of inosinic acid in 1 per cent hydrochloric acid, it is hydrolyzed to hypoxanthine and *d*-ribose phosphoric acid. The structure of this ribose phosphoric acid is readily shown by oxidation with nitric acid, phosphoribonic acid being obtained. Since, by this treatment, the pyranose form of ribose phosphate (whether substituted in positions 2, 3 or 4) would give rise to a phosphoribotrihydroxyglutaric acid, it follows that position 5 of the sugar chain is protected by the phosphoryl group and that ribose phosphoric acid is 5-phosphoribofuranose. This conclusion was subsequently confirmed by an investigation of the rate of lactone formation. The transformation of the phosphoribonic acid to its lactone was found to proceed very slowly, a property characteristic of γ -lactones. The formation of δ -lactone was not observed, as was to be expected if position 5 of the sugar is substituted.

The discovery that adenosine and inosine are ribofuranosides⁶ obviously further substantiates this formulation, and the question was finally settled beyond doubt by the recent synthesis of 5-phosphoribose.¹¹ It had been discovered that ribose condenses with a mixture of methyl alcohol and acetone in the presence of sulfuric acid and anhydrous copper sulfate to give 2:3-monoacetone methylribofuranoside having a free hydroxyl at position 5 only. This was phosphorylated and the acetone and glycosidic groups were hydrolyzed, yielding 5-phosphoribose (I) identical with the natural substance.

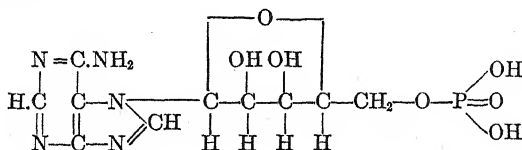


I.

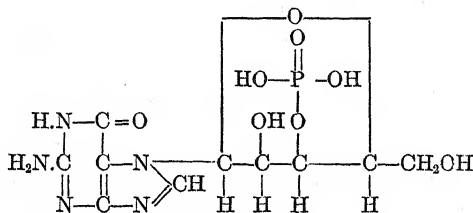
It follows that inosinic acid is 7'-hypoxanthine-5-phosphoribofuranoside:



Muscle Adenylic Acid.—Embden¹² discovered in muscle extracts an adenylic acid which has since been isolated from heart muscle and from the brain. The presence in muscle tissue of an inosinic acid immediately suggested a possible relationship between it and muscle adenylic acid. Schmidt¹³ established this relationship, isolating from muscle tissue an enzyme capable of transforming the adenylic to the inosinic acid. It was further shown that the two acids had the same rate of hydrolysis. Muscle adenylic acid is therefore 7'-adenine-5-phosphoribofuranoside:



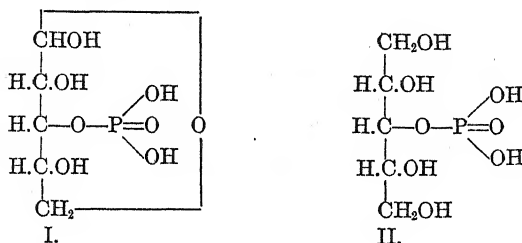
Guanylic Acid.—By neutral or slightly alkaline hydrolysis the guanylic acid from yeast gives guanosine and phosphoric acid. On deamination by means of nitrous acid, it is transformed to xanthylic acid, the structure of which is discussed in the next section. Guanylic acid may therefore be formulated as 7'-guanine-3-phosphoribofuranoside:



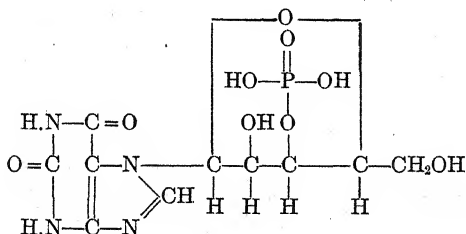
In 1893 Hammarsten¹⁴ obtained the first indication of the presence in animal tissues of a nucleic acid different from thymonucleic acid. Bang¹⁵ isolated from the pancreas a substance which he termed *guanylic acid*, since on hydrolysis it gave rise to guanine, phosphoric acid and a pentose (later identified as *D*-ribose). Steudel showed that on boiling the pancreas with water, the extract contains guanylic acid and the residue thymonucleic acid. Guanylic acid has also been isolated from the spleen and the liver. Since this acid has the same rate of hydrolysis as the yeast guanylic acid, it is supposed that the two are identical.

Yeast Xanthylic Acid.—Yeast xanthylic acid, obtained by the deamination of yeast guanylic acid, is readily hydrolyzed in aqueous solution at its own pH (1.9) with the formation of xanthine and a ribose phosphoric acid.¹⁶ The properties of this ribose phosphoric acid differ markedly from those of 5-phosphoribose from muscle inosinic acid. In the first place, 5-phosphoribose condenses with methyl alcohol in the presence of dry hydrogen chloride to give a furanoside only,

whereas the new phosphoribose yields both the furanoside and pyranoside forms of the glycoside. The two phosphoriboses can be cautiously oxidized, giving the corresponding phosphoribonic acids. The phosphoryl group is hydrolyzed twice as rapidly from the new phosphoribonic acid as from 5-phosphoribonic acid, and polarimetric observation of the rate of lactone formation in the new phosphoribonic acid shows marked difference from that of its isomer. By reduction with hydrogen in the presence of a suitable catalyst, the new phosphoribose is transformed to inactive phosphoribitol (II). It follows that the new phosphoribose is 3-phosphoribose (I).

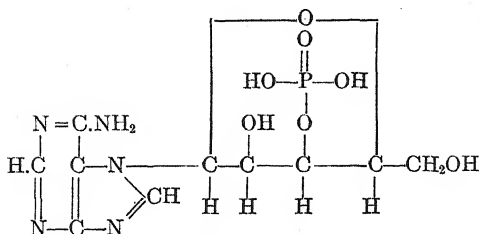


Yeast xanthylic acid is therefore 7'-xanthine-3-phosphoribofuranoside:

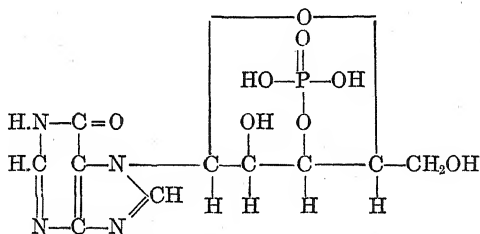


Yeast Adenylic Acid.—Yeast adenylic acid is obtained by gentle hydrolysis of yeast nucleic acid. On further hydrolysis with ammonia, it gives adenosine and phosphoric acid. Schmidt's enzyme, which deaminizes muscle adenylic acid, has no effect on yeast adenylic acid, and, furthermore, the two acids differ in melting point and in specific rotation. Under the same experimental conditions, yeast adenylic acid is hydrolyzed to the extent of 50 per cent in two hours, whereas muscle adenylic acid requires fifteen hours to reach the same degree of hydrolysis. Yeast adenylic acid is, therefore, an isomer of muscle adenylic acid and cannot have the phosphoryl group situated at position 5 of the ribose residue. It is nevertheless a furanoside derivative, since it is readily hydrolyzed to adenosine.

Levene and Harris¹⁷ have succeeded in deaminizing yeast adenylic acid by means of nitrous acid to give yeast inosinic acid, the structure of which is discussed in the next section. Yeast adenylic acid should therefore be designated 7'-adenine-3-phosphoribofuranoside:



Yeast Inosinic Acid.—Yeast inosinic acid, obtained by the deamination of yeast adenylic acid, is readily hydrolyzed in aqueous solution at its own pH to adenine and a ribose phosphoric acid. Levene and Harris¹⁷ have compared the properties of this acid with those of the acid obtained from yeast xanthylic acid. They had the same specific rotation in water (which was enhanced to the same extent in the presence of borax), and the same rate of hydrolysis by dilute mineral acids to give ribose and free phosphoric acid. Thus, the ribose phosphoric acid from yeast inosinic acid is 3-phosphoribose and yeast inosinic acid may be designated as 7'-hypoxanthine-3-phosphoribofuranoside:

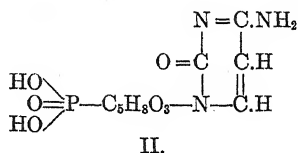
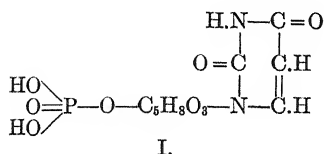


Pyrimidine Ribonucleotides.—Like their parent nucleosides, uridylic and cytidylic acids give only a faint orcinol test. In addition to the stability of the glycosidic union, the phosphoryl group adheres to the pentose much more tenaciously than in the purine ribonucleotides. Hydrogenation, however, gives dihydro-uridylic and dihydro-cytidylic acids, from which both phosphoric acid and base are readily hydrolyzed. The rate of hydrolysis of the phosphoryl group is, indeed, comparable with that of the 3-phosphoribosides.

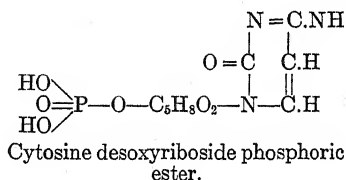
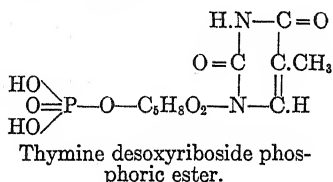
Owing to their resistance to acid hydrolysis, a mixture of uridylic and cytidylic acids is readily obtained by hydrolyzing yeast nucleic acid with 2 per cent sulfuric acid, the purine nucleotides being destroyed. The two nucleotides are separated by conversion to the brucine salts which are fractionally crystallized.

Since dihydrocytidylic acid is hydrolyzed at a rate comparable with that of yeast adenylic acid, it seemed probable that the phosphoryl group is not attached at position 5 of the ribose component. This was confirmed by the synthesis of uridine-5-phosphoric acid (by the phosphorylation of mono-acetone uridine),¹⁸ a substance having properties quite different from those of yeast uridylic acid. The structures

of uridylic (I) and cytidylic (II) acids may be represented provisionally by the following formulae:

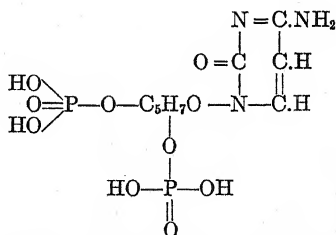
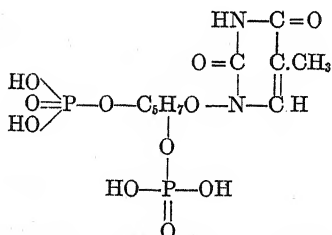


The Structure of the Desoxyribose Nucleotides.—The problem of finding a method of hydrolyzing thymonucleic acid in such a way that the four nucleotides may be isolated intact has not yet been solved. Only the two pyrimidine derivatives have been obtained—owing to their greater stability towards hydrolyzing agents than the purine desoxyribose nucleotides. Their formulae may be provisionally represented as follows:



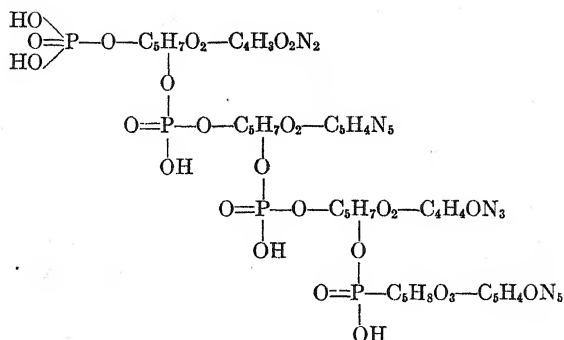
Diphosphoric esters of thymine and cytosine desoxyribosides have also been isolated, by hydrolysis of thymonucleic acid with 2 per cent sulfuric acid. The isolation of these derivatives has helped in elucidating the structure of thymonucleic acid. On catalytic hydrogenation, the thymine diphosphodesoxyriboside gave a product readily hydrolyzed by 2 per cent sulfuric acid to thymine and a phosphoric ester which was reduced by boiling Fehling's solution.

These two nucleotides may therefore be represented as:



POLYNUCLEOTIDES

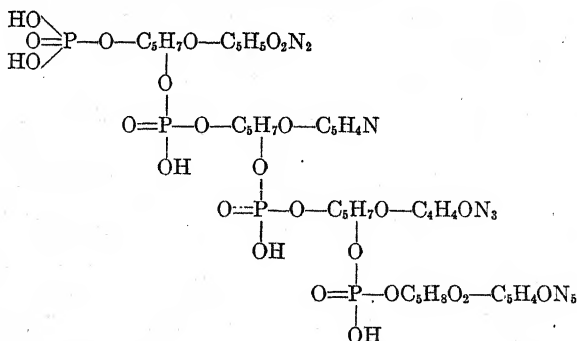
Yeast Nucleic Acid (Tetranucleotide).—Since mild hydrolysis of yeast nucleic acid gives directly the four mononucleotides, it has been formulated by Levene as a tetranucleotide, in which there is an ester linkage between the individual nucleotide residues:



Many other structures have been proposed from time to time, but as the above is the only one which is in conformity with the observed physicochemical properties of yeast nucleic acid, the other formulae may be ignored.

Animal Ribose Nucleic Acid.—The discovery of the occurrence of guanylic acid in animal tissues was succeeded by the work of Jones and Perkins¹⁹ who obtained three ribonucleotides—guanylic, adenylic and cytidylic acids—by the alkaline hydrolysis of pancreas nucleoprotein. Jorpes²⁰ showed definitely that animal tissues contain, in addition to desoxyribose nucleic acid, a nucleic acid of the ribose type. There is, however, lack of agreement as to whether this acid is a penta- or a hexanucleotide.

The Structure of Desoxyribose Nucleic Acid.—Since partial hydrolysis of thymonucleic acid gives rise to four nucleosides and complete hydrolysis gives the four bases in equimolecular proportions, the acid may be regarded as a tetranucleotide:



Other formulations proposed may be dismissed as contrary to the physicochemical properties of thymonucleic acid. In the above formula, having alternating purine and pyrimidine residues, the isolation of the diphosphoric esters of the 2-pyrimidine desoxyribosides is accounted for.

R. STUART TIPSON.

REFERENCES

1. Miescher, F.: *Hoppe-Seyler's Med.-chem. Unters.*, 441 (1871).
2. Hoppe-Seyler, F.: *Hoppe-Seyler's Med.-chem. Unters.*, 486 (1871).
3. Altmann, R.: *Arch. für Physiol.*, 524 (1889).
4. Levene, P. A., and La Forge, F. B.: *Ber.*, 43, 3164 (1910).
5. Levene, P. A.: *J. Biol. Chem.*, 53, 441 (1922).
6. Levene, P. A., and Tipson, R. S.: *J. Biol. Chem.*, 94, 809 (1932); 97, 491 (1932).
7. Levene, P. A., and Tipson, R. S.: *J. Biol. Chem.*, 104, 385 (1934).
8. Levene, P. A., and Tipson, R. S.: *J. Biol. Chem.*, 101, 529 (1933).
9. Levene, P. A., and London, E. S.: *J. Biol. Chem.*, 81, 711 (1929); 83, 793 (1929).
- 9A. Levene, P. A., and Mori, T.: *J. Biol. Chem.*, 83, 803, (1929).
10. Liebig, J.: *Ann.*, 62, 257 (1847).
11. Levene, P. A., and Stiller, E. T.: *J. Biol. Chem.*, 104, 299 (1934).
12. Embden, G., and Zimmermann, M.: *Z. physiol. Chem.*, 167, 137 (1927).
13. Schmidt, G.: *Z. physiol. Chem.*, 179, 243 (1928).
14. Hammarsten, O.: *Z. physiol. Chem.*, 19, 19 (1894).
15. Bang, I.: *Z. physiol. Chem.*, 26, 133 (1898-1899).
16. Levene, P. A., and Harris, S. A.: *J. Biol. Chem.*, 95, 755 (1932); 98, 9 (1932).
17. Levene, P. A., and Harris, S. A.: *J. Biol. Chem.*, 101, 419 (1933).
18. Levene, P. A., and Tipson, R. S.: *J. Biol. Chem.*, 106, 113 (1934).
19. Jones, W., and Perkins, M. E.: *J. Biol. Chem.*, 62, 291 (1924-1925).
20. Jorpes, E.: *Acta med. Scand.*, 68, 503 (1928).

Suggested Reading

- Levene, P. A., and Bass, L. W.: *Nucleic Acids* (1931).
Jones, W.: *Nucleic Acids* (1920).

CHAPTER VIII

NUTRITION

PRIMITIVE man, whenever possible, ate such foods as he could find, making his selection on the basis of taste and smell. When faced with famine conditions hunger succeeded appetite, and he ate anything edible even though unappetizing. Palatability was the early basis for judging quality in food. Under the settled conditions of civilization, the food supply became in favored regions so abundant and varied as to afford an opportunity for great refinement of culinary practice. There has been maintained an intimate relation between civilization and cookery which is reflected in a long series of cook books. The earliest of these of which we have any record is that of Athenaeus, written about 200 A. D.,¹ in which a company of gentlemen guests at a banquet discuss their views concerning many foods and beverages after the style of the Symposium of Plato. Modern refinement has produced books such as that of Sheridan's *The Stag Cook Book*, advertised as telling of "sauces from the South, chowders from New England, barbecued masterpieces from the West, grilled classics from field and stream, ragouts, stews, desserts, dressings, . . . hung within the reach of all, like garlic clusters from the rafters of opportunity."

The earliest scientific interest in nutrition appears to have been centered on the nature and extent of the process of digestion. Physiologists and biochemists are still seeking to learn more about the secretion of the digestive fluids (their action on proteins, lipoids, carbohydrates, nucleic acids, etc.), and the processes of absorption. Much mystery remains to be cleared up in this field.

Energy Metabolism.—Since from the quantitative standpoint the greatest nutritional demand is for energy, it is not surprising that at one time the principal emphasis in investigation was placed upon the provision of fuel sufficient to furnish the energy for work and heat. Only a few years ago the comparison of the body to an engine, and of the food to fuel seemed to many an adequate one. The more nutrition is studied the greater is the appreciation of the extraordinary complexity of the chemical processes associated with the phenomena of life. Investigation of energy metabolism yielded valuable knowledge concerning the basal metabolic rate, and also the energy needs of the body during various kinds of physical activity. The caloric value of all the more important natural foods was studied and the results form a permanent contribution to our knowledge of nutrition.²

The Biological Value of Proteins.—Metabolism studies early revealed that there is a constant disintegration of the highly organized

structures which are generally designated as protoplasm and which form the physical basis of life. This necessitates a continual flow of new nutrient material through the tissues, so that constant repair may take place. In the growing organism this repair goes on simultaneously with increase in amount of body substance which constitutes growth. From a quantitative standpoint the greatest need in this respect is for protein. Since advancing knowledge of proteins revealed that they all yield a series of at least twenty-two amino acids, the proportions differing widely in proteins from different sources, corresponding changes in viewpoint concerning the protein element in nutrition were inevitable. Less than half a century ago proteins from various sources were generally believed to have essentially the same nutritive values. By 1900 it was clearly evident that the proteins differed so widely in their composition that there must be differences in their nutritive values. Modern studies have increasingly emphasized the importance of this discovery. Many quantitative studies have been made which show the approximate "biological values" of proteins from different sources.

The development about sixty years ago of methods for the chemical analysis of foodstuffs aided greatly in the visualizing of their composition. It was found that almost the entire matter in a food sample could be accounted for in terms of proteins, carbohydrates, lipoids and ash; consequently the more credulous and less inquiring minds tended to accept the view that these were the sole important constituents of foods from the nutritional standpoint. The pigments and other extractives were ignored as of no significance. But the more curious and reflective students of nutrition long ago asked themselves more specific questions about foods and the processes of metabolism. The demonstration of the differences in the yields of amino acids by different proteins showed that all proteins taken as food are foreign bodies and not suited for direct incorporation into the body tissues. This idea led to revolutionary views of digestion. Food proteins were shown to be resolved into their constituent amino acids, which were then absorbed and circulated in the body fluids, from which each tissue abstracted its quota and recombined them into new proteins having different proportions and arrangements of amino acids in their molecules. Now the demonstration that digestion of muscle proteins in a healthy stomach forms some substance which is essential to the normal hemopoietic function, whereas in the digestion in the stomach of a subject with pernicious anemia it is not formed, seems to necessitate a revision of viewpoint. The assumption must be made that certain peptides escape digestion, and that their unique structure is of fundamental significance in nutrition. The constant presence of glutathione, a tripeptide, in almost all tissues, suggests the same viewpoint. We do not know whether the latter is synthesized by the living tissues or whether it is a fragment of protein which escapes normal digestion and enters the blood from the alimentary tract.

Essential Amino Acids.—From the standpoint of an understanding of body chemistry, a field of inquiry of singular interest is that relating to the capacity of the body to synthesize the amino acids derivable through hydrolysis of proteins. Osborne and Mendel³ have demonstrated that histidine, lysine, cystine and tryptophan are indispensable components of the diet. Tyrosine and phenylalanine (Rose⁴) appear also to be necessary. On the other hand the evidence supports the view that arginine, aspartic, glutamic and hydroxyglutamic acids, proline and hydroxyproline, glycine and probably alanine, are not necessary in the diet because they can be synthesized in the body; but from what precursors it is not known. The list of indispensable amino acids is yielded by all ordinary food proteins. There is no reason to believe that at any time in evolutionary history there arose a need for the synthesis of these building stones of proteins. Living tissues have acquired the power to make them, but are still dependent upon an exogenous source of the indispensable variety.

Although it is not possible at present to analyze proteins for their amino acid content by chemical methods, it is a simple matter to feed the proteins of a single foodstuff or of combinations of two or more, and to determine the "biological value" and the supplemental values of proteins from different sources (by observing the rate of growth at different levels of protein intake, or maintenance at very low levels of protein feeding, etc.). A valuable body of such evidence is now available.

The Nucleoproteins.—There is no positive evidence from experiments on animals which indicates that the purines and pyrimidines, or any nucleoside or nucleotide derivable from the nucleic acids has any significance as a constituent of the diet. It appears that these substances are all synthesized to form nucleic acids during cell activity, and that the nuclear components derived from food are all destroyed. It must be added, however, that so long as we must rely upon extracts to supply the water-soluble vitamins of the *B* series, there is the possibility that the above statements may need revision. This question will be settled when pure vitamins are available for experiments with a diet of purified foodstuffs.

The Carbohydrates.—There are no obvious problems involving carbohydrates in the nutritional field, apart from the present impossibility of ridding starches from their small content of lipoids. The presence of these lipoids leads to uncertainty in interpreting the results of studies with purified foodstuffs when starch or dextrin is included in the diet. The interest and importance of the problems of carbohydrate metabolism lie in the field of intermediate metabolism. The evidence all points to the probability that any source of carbohydrate in the diet which provides a source of glucose will prove adequate. The ready synthesis of galactose from glucose in milk elaboration and for the synthesis of the galactosides of the nervous tissues is well known

The Lipoids.—The capacity of the body to synthesize fatty acids of many kinds, glycerol, lecithin, kephalin, cholesterol and probably ergosterol, as well as the cerebrosides, creatine and its phosphoric acid ester, glucose phosphoric acid esters, etc., illustrates the extraordinary synthetic capacity of the body. The biological chemists will continue to seek to discover which of the chemical substances which play a rôle in body chemistry are capable of synthesis, and which must be provided in the food. It appears from the studies of Burr⁵ that linoleic acid or linolenic acid is necessary in the diet; and that either will serve as the sole indispensable representative of the lipoids. This statement is based upon the assumption of the correctness of the view that unactivated ergosterol is not absorbable from the alimentary tract. If this is true, then the ergosterol which is present in all tissues and which makes them susceptible to activation in the sense of becoming antirachitic on irradiation with ultraviolet light, must originate in the body through synthesis. This field is still new and it may well be that the viewpoint here presented may be modified by future study.

The Inorganic Elements.—Among the inorganic elements, it has been satisfactorily demonstrated that at least eleven are indispensable for adequate nutrition. These are sodium, potassium, calcium, magnesium, chlorine, iodine, phosphorus, sulfur, iron, copper and manganese. The amount of each of these elements which suffices in the diet of a mammal has not been determined. Indeed, there is evidence that the requirements of one may in certain instances be determined by the relative abundance of one or more others (*e. g.*, the importance of a suitable calcium to phosphorus ratio).

Response to deprivation of the different inorganic elements is remarkably different. Feeding young rats a diet lacking vitamin D and containing unfavorable ratios of calcium and phosphorus, the level of intake being under 1.2 per cent of the food, results in severe and acute rickets in nineteen to twenty-one days. Feeding diets too poor in both calcium and phosphorus affects the structure of the bones, especially if the supply of vitamin D or of ultraviolet rays is cut off. On the other hand, deprivation of chlorine or sodium does not soon result in the appearance of symptoms indicating the development of a pathologic state. Under such conditions, excretion of the missing elements is reduced practically to zero. In the case of deprivation of sodium or potassium, it is necessary to remove almost the last trace of the element from the food and wait several weeks before signs of failure become manifest.

In striking contrast to the delayed response to deprivation of sodium, potassium or chlorine, stands the prompt failure with striking symptoms when the magnesium content of the diet is reduced to about 1.8 parts per million. Young rats pass within three to five days into a condition of hyperemia of the skin which increases from the eleventh to the fourteenth day when it begins to subside, and is followed by pallor and finally by cyanosis. There is marked increase in irritability from

the beginning of the hyperemia of the vascular bed until the eighteenth day, when any sudden disturbance throws the animal into a fright which is followed by a convulsive seizure, in which about 80 per cent of the rats die. There is an early and progressive decrease in the magnesium content of the blood, and a marked increase in the content of cholesterol esters.⁶

Normal animals steadily deprived of iron develop anemia. When fed on milk exclusively, severe anemia develops in a few weeks.⁷ Normal animals deprived of iodine require many weeks to bring on symptoms of iodine deficiency resulting from shortage of thyroxine. Rats deprived of manganese show abnormality in males in injury to the germinal epithelium in about one hundred days. In females, deficiency of manganese does not interfere with fertility but apparently makes normal lactation impossible; and about the same period of deprivation as in the male is required to bring about this effect. Copper is essential for the utilization of iron for the synthesis of hemoglobin.⁷

The vitamin deficiency diseases are discussed in the next chapter.

The Adequate Diet.—We may, in the light of the facts set forth, visualize an adequate diet as one providing an unknown number of amino acids. Six have been shown to be indispensable, and seven of the twenty-two known hydrolytic products of proteins have apparently been proved to be capable of synthesis. If we postulate the necessary provision of the fifteen amino acids which have not been shown to be capable of synthesis by the body, the known dietary essentials, in their simplest forms, consist of these, together with eleven inorganic elements in utilizable forms, six vitamins, linoleic acid and a source of glucose other than that which can be formed in the body from protein and the glycerol moiety of fats. We have then thirty-four simple chemical nutrients which form the pabulum with which all the complex chemical processes of metabolism are possible. Advances in our knowledge have been so rapid in recent years that it would be unwise to conclude that our knowledge is complete. It is not improbable that new amino acids will be discovered among the products of hydrolysis of proteins. Already there are claims in the literature of the existence of nine or ten vitamins. It is not at all improbable that inorganic elements other than those listed here will be shown by further research to play physiologic rôles. What we know about the nutritive needs of the body we know in terms of the thirty-four simple nutrients named, and what we know about quality in foods we know in terms of what each provides in the same nutrients. The methods of experiment by means of which further progress is to be expected are well defined, and with the rapid growth of enthusiasm for investigation of nutritional problems, we may look forward with confidence to the attainment of an extraordinarily complete knowledge of the subject of nutrition within the next one or two decades. Further progress is to be anticipated along the lines mentioned, and likewise in the study of the

interconvertibility and the chemistry of the intermediary metabolism of foodstuffs, the chemical reactions concerned with the exchange of energy, the nature of the precursors among the nutrients of the hormones and enzymes, and the physicochemical processes involved in physiology.

Nutrition Problems of Man.—Before the development of the science of nutrition, there existed in many parts of the world human nutrition problems the nature of which could not be understood. The occurrence of vitamin A deficiency in remote times is clearly shown by the reference to night blindness in the Ebers Papyrus written about 1500 B. C. There the passage occurs which states that "Because the unknown disease was cured by the roast liver of an ox, the disease was supposed to be night blindness. Also the patients were recommended to hold their heads over the steam rising from roasting liver. By early writers the liver of a black cock was also recommended." Who were these early writers and how far back did they flourish? Today the Newfoundland fishermen subsist on a diet which in some cases causes beriberi and also night blindness. Working throughout a bright day in the glare on the water, the visual purple in the retina is bleached and is not resynthesized. In the twilight or darkness of a starlit night the man becomes practically blind. Impairment of the integrity of the mucous membranes due to lack of vitamin A or its precursor, carotene, reduces their effectiveness as a barrier against bacterial infections. The importance of vitamin A deficiency as a human health problem cannot be accurately appraised. Pronounced avitaminosis A as observed in Denmark during the World War was caused by the exportation of too much of milk fat as butter. Night blindness is relatively rare, but its occurrence in many parts of the world suggests undernutrition with respect to this nutrient.

Deficiency of vitamin B₁ constitutes one of the major health problems of the world. In all rice-eating nations this is the cereal of choice for one reason only. It is the cereal of the swamp and of hot, moist climates, and the only one which can be grown successfully. Unpolished rice becomes rancid easily; hence the practice of polishing the grain is very old. In this process the vitamins are essentially all removed. Polished rice is a satisfactory energy food, but is very deficient in most of the nutrients other than starch. The practical problem is to supplement polished rice with foods which make good its deficiencies. Beriberi is probably seventh or eighth among the causes of ill health in tropical and subtropical regions. We have abundant knowledge to enable us to eradicate the disease from the face of the earth within a few years if it could be applied. It is purely an economic problem.

Scurvy is by no means so common as formerly. It has been the scourge of sailors and soldiers in the not very distant past. Today it is in great measure restricted to infants which are bottle-fed with heated milk. It is so easily prevented and at so little cost, that education of mothers has tended greatly to reduce its incidence.

Rickets is still of frequent occurrence, but its incidence has been greatly reduced through the spread of knowledge of the prophylactic efficiency of cod liver oil and viosterol. The latter is vitamin D produced by irradiation of ergosterol obtained from yeast. The prevalence of rickets among infants and children is due to the high cost of either of these sources of the vitamin.

Pellagra is a national health problem in the United States and a few other places. Approximately 200,000 persons are now suffering from the disease in this country because of inadequate diet.

The Diet and Tooth Decay.—Tooth decay is the cause of an enormous amount of physical discomfort and of ill health. The decayed tooth often becomes abscessed, and from this focus the blood stream is polluted and secondary foci may be established in the gall-bladder, joints, heart, kidney or elsewhere. Decayed teeth are probably a lesser menace than infected tonsils, but they are a great menace to health. In recent years there has been an increasing interest among nutritional investigators in the relation of the diet to tooth decay. Several viewpoints have emerged from these researches: Mellanby¹³ emphasizes the protection of children against dental caries induced by the provision of vitamin D. Her researches have established (as have those of Kline and McCollum) the importance of an adequate supply of vitamin D for the development of teeth which are free from enamel defects. In studies on children (at Birmingham, England) who were fed a diet which appears adequate by ordinary standards, she was able greatly to reduce the incidence of caries either by giving cod liver oil or "radiosterol"—the equivalent of our viosterol.

McCollum has emphasized the fact that the herbivora normally eat food containing two to six times as much total mineral matter as is supplied by a diet of whole cereals, and that the carnivora all eat much calcium phosphate if they have access to bones. People nearly everywhere are subsisting on a very low supply of mineral elements, especially calcium and phosphorus.¹⁴ It is apparent that where the diet derives at least 30 per cent of its caloric value from refined cereal products, and 15 to 20 per cent from sugars, the ash content must be far below that which man has been accustomed to take before the advent of modern machinery for milling, and before sugar was manufactured on a large scale. These foods are not to be summarily condemned, but great emphasis should be laid upon inclusion in the diet of such supplemental foods as will correct these deficiencies.

Bunting¹⁵ has emphasized the relation between *B. acidophilus* as the prevailing mouth flora and the incidence of dental caries. A strong argument against the overemphasis of this theory of the etiology of caries of the teeth is the reported excellence of the teeth among sour milk-drinking peoples in pastoral regions. This view of the caries problem cannot be appraised accurately on the evidence available.

Hanke¹⁵ has reported excellent results in the control of caries of the teeth by the daily consumption by his patients of very large

amounts of citrous fruit juices. He postulates the protective action as due to the high intake of ascorbic acid. The Eskimos, however, and the people of the island of Tristan da Cubna in the South Atlantic ocean, are exceptionally free from disease although they have a relatively low vitamin C intake. Hanke ignores the inorganic moiety of the generous ration of fruit juice.

The prevention of tooth decay doubtless will be achieved when children are so nourished as to develop teeth without structural defects, and thereafter subsist upon a dietary which maintains the blood chemistry at the optimum in respect to calcium and phosphate ions and buffer value. The blood chemistry will be reflected in the character of the saliva, which, if of optimum quality in respect to these chemical factors, will go far to protect the enamel from disintegration, and to neutralize any acids which may be formed through fermentation of carbohydrate residues on the teeth. But any dietary which permits of mouth conditions in which food débris form undisturbed areas on the teeth, covered by fermentable carbohydrate, will result in etching and eventual undermining of the enamel and the establishment of dental caries.

Of the several views as to the cause of dental caries, each appears to have some merit, but each seems to be too narrow. There is no question, however, that much can be done, through adequate nutrition, to reduce the incidence of caries of the teeth.

Nutritional Problems of Animal Production.—Space does not permit an adequate discussion of the extent to which inadequate food in animal production throughout the world results in economic loss. The greatest single problem appears to be due to shortage of phosphorus in many semi-arid grazing areas. This is well known to be true of vast areas in South Africa and Australia, and in many other local areas of lesser extent. In thirty counties in Minnesota, farm animals are unthrifty because of deficiency of phosphorus in the soil and the crops grown on them.³ In New Zealand, Florida and a few other places there is deficiency of iron in the locally grown foods with consequent anemia. In parts of Holland, and in New Zealand, deficiency of magnesium appears to be the cause of "grass tetany," which causes the death of many cows in early lactation. Milk fever in lactating cows and the "lambling sickness" from which many ewes die are now known to be caused by calcium deficiency. In the aggregate, the application of recent scientific discoveries to the feeding of farm animals would result in an enormous economic gain.

The Protective Foods.—Modern researches have shown that nearly all foods are deficient in one or another nutrient, or are not well balanced in certain elements from the standpoint of physiologic needs. We no longer condemn any food on the basis of its deficiencies, but accept it for what it is and recommend its use in combination with supplemental foods so constituted as to make good its deficiencies. All cereals and their milling products, tubers, roots, and fruits are

unbalanced in several respects from the dietary standpoint.³ The same may be said of the lean meats. Even milk, a nearly perfect food, is too poor in iron and copper. The contents of the egg shell are too poor in calcium, which the chick derives from the shell. The foods very nearly perfect for animals are the grasses and other leaves of plants. These are not suitable for human consumption except in small amounts. The supplemental foods of outstanding value are milk, eggs, meats and the leafy vegetables. None of these will supply a liberal amount of ascorbic acid; therefore, some raw or commercially canned fruits or vegetables should always appear in the daily menu. In general, one may say that the more liberally the supplemental foods named are consumed the more adequate will the human diet be. These have been designated the *protective foods* because of their special value in providing the indispensable nutrients.

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REFERENCES

1. *Athenaeus* in the Loeb Classical Library.
2. Lusk, G.: *Nutrition* (1933).
3. McCollum and Simmonds: *The Newer Knowledge of Nutrition* (1929).
4. Rose, W. C.: *Proc. Amer. Soc. Biol. Chem.*, **7**, 66 (1931).
5. Burr, G. O., and Burr, M. M.: *J. Biol. Chem.*, **82**, 345 (1929).
6. Kruse, H. D., Orent, E. R., and McCollum, E. V.: *J. Biol. Chem.*, **96**, 519 (1932); **100**, 603 (1933).
7. Waddell, J., Steenbock, H., and Hart, E. B.: *J. Biol. Chem.*, **77**, 769 (1928).
8. Mendel, L. B.: *J. Am. Med. Assoc.*, **98**, 1981 (1932).
9. Kruse, H. D., and McCollum, E. V.: *J. Am. Med. Assoc.*, **98**, 2201 (1932).
10. Levene, P. A., and Raymond, A. L.: *Science*, **78**, 64 (1933).
11. King, C. G., and Waugh, W. A.: *Science*, **75**, 357 (1932).
12. Hess, A. F.: *J. Am. Med. Assoc.*, **98**, 1429 (1932).
13. Mellanby, M.: *Lancet*, ii, 767 (1918); *Brit. Med. J.*, ii, 354, 1079 (1924).
14. McCollum, E. V.: *Proc. First District Dental Soc.*, *New York Journal of Dentistry*, **4**, 9 (1934).
15. Hanke, M. T.: *Diet and Dental Health* (1933).
16. Bunting, R. W., et al.: *Dental Cosmos*, **68**, 931 (1926).

CHAPTER IX

THE VITAMINS

Historical Introduction.—It is interesting to compare the phases traceable in the history of almost every great intellectual movement with those which together constitute the characteristic "growth curve" of living organisms.

The freshly implanted culture of yeast exhibits four clearly marked phases. The short "lag-phase" introduces the period of rapid growth and full cellular activity. Then, as the supplies of nutriment are reduced and as the end-products accumulate, the activity of the colony falls off, and a period of stagnation or decline caused by starvation and by chronic poisoning sets in. Are not these same phases clearly apparent in the records of our political and religious systems and in the history of the acquisition of scientific knowledge?

When the twentieth century opened the science of nutrition was in a state of quiescence. The cycle was almost complete. It had begun a hundred years earlier with the remarkable outburst of energy represented by the work of Lavoisier. For a time the curve ran steeply, sustained by the vigor of mind of Liebig, but by the seventies there is clear evidence that a large proportion of the energy of the original impetus had been dissipated. The work of Pettenkofer, Voit, Atwater and of many other bearers of honored names covers a period of quiet consolidation which marks the later stages of the cycle. New ideas were needed; the culture must be renewed. It is easy for us to appreciate how the new movement began. The material achievement of the cycle just described was the acquisition of knowledge regarding the quantitative requirements of the living body for tissue-forming components and for energy. The views expressed in any one of the many textbooks of physiology published about this time can be represented by what we find in Halliburton's well-known volume in 1905. "The chief chemical compounds or *proximate principles* in food are:

- | | |
|------------------|---------------------|
| 1. Proteids | } <i>organic.</i> |
| 2. Carbohydrates | |
| 3. Fats | |
| 4. Salts | } <i>inorganic.</i> |
| 5. Water | |

"A healthy and suitable diet must possess the following characters:

"1. It must contain the proper amount and proportion of the various proximate principles.

"2. It must be adapted to the climate, to the age of the individual and to the amount of work done by him.

"3. The food must contain not only the necessary amount of proximate principles but these must be present in a digestible form."

Opinion differed as to what could be regarded as an "average daily diet" so that there was a liberal range from which to choose. Typical of these diets are the two given below:

| | <i>Moleschott</i> | <i>Voit</i> |
|-------------------|-------------------|-------------|
| Proteid..... | 120 | 118 |
| Fat..... | 90 | 56 |
| Carbohydrate..... | 333 | 500 |
| Calories..... | 2694 | 3055 |

It was inevitable that these theories should be put to a severe test, particularly since advances in the field of chemistry had made it possible to prepare a large number of "proximate principles" of foods in a state of reasonable purity. Actually, the first record of a suspicion that there might exist dietary components of the type recognized today as the vitamins is to be found in one of the series of papers which were published from the famous laboratory of Professor v. Bunge at Basle, where researches on the function of inorganic salts in foods were actively in progress. As far back as 1881, in a paper entitled "Ueber die Bedeutung der anorganischen Salze für die Ernährung des Thieres," Lunin had reported that young mice were unable to thrive on an artificial mixture compounded of the "proximate principles" of milk. He expressed the view that his results might be interpreted by assuming the existence of "unknown substances" without which life could not be sustained on a mixture of proteins, fats, carbohydrates and salts.

There is no record of attempts having been made to explore in v. Bunge's laboratory the interesting question raised by the suggestion in Lunin's paper; a suggestion which attracted curiously little attention elsewhere and which gradually was forgotten. To some extent it was ignored because orthodox thought preferred to accept what appeared to be a simpler and more reasonable explanation of the nutritive failure of the experimental animals. This was that the purified diets were so unpalatable and monotonous that loss of appetite and malnourishment were inevitable.

The first to show that this explanation would not serve and that Lunin's suggestion of the existence of unknown dietary factors might be seriously entertained was Professor Pekelharing of Utrecht.*

This famous teacher had visited Batavia in 1887 where he had seen the early stages of the brilliant series of investigations which Eijkman and his colleagues made on beriberi. By 1896, when he came

* I am greatly indebted to Professor G. Grijns of the Agricultural High School, Wageningen, for full details of the early experiments of Professor Pekelharing. He also very kindly provided me with a translation of the passage from the account of Pekelharing's lecture published in 1905 (*Nederl. Tijdschrift v. Geneeskunde*, 51, 111) which I have given.

on leave to Europe, Eijkman was satisfied that there is a substance present in the outer coats of the rice grain which is essential for health. He himself believed that when this substance is absent from the diet, carbohydrate breakdown may follow an abnormal path; a view to which, curiously enough, we are tending to return after nearly forty years.

One can well imagine the discussions in the laboratory at Utrecht, stimulated by correspondence with other colleagues, such as Grijns, who were pushing forward the investigations in the Dutch Indies, which led Pekelharing to attempt again the task of rearing young animals on artificial diets composed of purified foodstuffs. The announcement of his results was made at a meeting of the "Nederlandsche Maatschappij for bevordering der Geneeskunst" on July 3, 1905, but his words hidden in a Dutch medical journal were unknown to few outside his own country.

"It has often been said that our food is adequate if it contains certain quantities of albumen, carbohydrates, fats and salts. Much discussion takes place whether it is possible to reduce the albumen below Voit's 118 Gm. and replace it by fat or carbohydrate; but little is said of other indispensable components of the food. . . . Yet physiology has demonstrated long since, that if food is to have its true value something more than these must be present. . . . It is impossible to keep an animal alive by feeding it with albumen, fat, carbohydrates, the necessary salts and water, *even if the quantities of these are amply sufficient*. . . . What has been published by other investigators I find confirmed by my own experiments. . . . It is, of course, very important for the science of nutrition to determine what substances other than albumen, fat and carbohydrates are necessary, but there is the difficulty. . . . Such a substance occurs in milk. . . . If white mice are fed on bread baked from casein, albumen, rice meal, a mixture of all the salts that should be present and if water is their only drink *they die from deficiency*. . . . At first all goes well. . . . The bread is eaten readily and the mice look healthy. . . . Soon they grow lean, their appetite diminishes and within four weeks all are dead. . . . But if milk is given instead of water they keep in health, *notwithstanding that the quantities of albumen, lactose and fat they take with the milk are insignificant by comparison with those in the bread they consume*.

"The component of the milk responsible for keeping them alive occurs in whey from which the casein and fat have been separated. . . . Hitherto, my repeated efforts over some years to separate this substance from whey and to gather further information about it have given disappointing results. . . . Therefore, I will not repeat them. . . . I only wish to emphasize that an unrecognized substance occurs in milk which is of paramount importance for nutrition even in minute quantities. . . . If it is lacking the organism loses the ability to utilize the well-known principal components of the food, appetite is lost *and with apparent abundance the animals die of starvation*."

A year later, Hopkins, speaking of his own experiment and conclusion before the Society of Public Analysts in London, opened up an even wider horizon. "In diseases such as rickets and scurvy, we have had for long years knowledge of a dietetic factor, but though we knew how to benefit these conditions empirically, the real errors in the diet are to this day quite obscure. . . . They are, however, certainly of the kind which comprises these minimal qualitative factors I am considering."

The "lag phase" occupied nearly ten years. Probably it would have taken longer to win general acceptance for views so unorthodox had the War not brought into prominence innumerable problems of human and animal nutrition which soon revealed themselves as concerning these very dietary factors. Today they are generally known as the *vitamins*: An unsatisfactory name, but one so universally employed that no useful purpose would be served by attempting at this late date to replace it by one more appropriate.

Vitamins.—One might reasonably define a vitamin as an essential constituent of the diet, organic in nature and effective in playing a part in the maintenance of the normal functioning of tissues in amounts minute by comparison with those of the foodstuffs which supply the structural material and the energy. In the early days of vitamin research they were frequently likened to the enzymes, because the idea was attractive that substances effective in such small amounts acted catalytically. Opinion has, however, veered round toward regarding them as more akin to the hormones. Indeed, there are some who prefer to term them the exogenous hormones, a name for which there is some justification, since it is not improbable that some of them are related to the activity of the glands of internal secretion. It is amusing to speculate how, during the long history of evolution, the animal organism has lost the power to synthesize certain molecular skeletons or to add certain groupings, so that in a curiously irregular and disconnected manner it exhibits absolute dependence on the plant.

Little more need be said regarding the history of the discovery of the vitamins. The effect of the small additions of milk observed by Lunin, Pekelharing and Hopkins was, before long, recognized to be due to the action of more than one essential substance. Osborne and Mendel, and simultaneously, McCollum and Davis, differentiated a *fat-soluble* factor clearly distinct from the water-soluble essential of the type which had been revealed by the researches on beriberi. Mainly as a result of rapid improvement in the technic employed for carrying out feeding experiments on small animals—some day a grateful world will offer a happy tribute to the white and piebald rat—the differentiation of other factors has proceeded apace. Today, no less than nine vitamins are recognized, and the existence of others is suspected. For no really satisfactory reason but rather for some slight convenience they are grouped under the old headings employed originally by McCollum: "fat-soluble" and "water-soluble."

FAT-SOLUBLE VITAMINS

On February 12, 1782, Robert Darbey, then house surgeon and apothecary to the Manchester Infirmary, wrote a letter to the keen-minded London clinician, Thomas Percival, which the latter has enshrined in one of his famous medical and philosophical essays. It was in answer to a request for information regarding the use of cod liver oil, then recently introduced as a therapeutic agent. "About ten years since, an accidental circumstance discovered to us a remedy, which has been used with the greatest success . . . but is very little known, in any country, except Lancashire. It is the cod, or ling liver oil."¹

The beneficial effect of fish liver oils in the treatment of rickets, osteomalacia, generalized malnourishment and certain eye conditions, was widely recognized by the middle of the nineteenth century, but in spite of much speculation no satisfactory theory to account for its superiority over other edible fats had been evolved. Not until 1913 did independent investigations by McCollum and Davis at Wisconsin and Osborne and Mendel at New Haven reveal by adequately controlled animal experiments that certain fats contain a factor essential for nutrition whereas others do not. Before twelve months had elapsed extension of the experiments at Wisconsin Station showed that the factor concerned resisted saponification and could be transferred as a constituent of the nonsaponifiable fraction to oils which formerly did not contain it.

The importance of this discovery was not only that it finally dispelled a curious variety of theories which had been current regarding the nature of the therapeutic agent in cod liver oil (*e. g.*, traces of iodine), but that it opened up a clear path for chemical attack on the properties and nature of the active substance. One factor to which the name "fat-soluble A" was given was believed to exist. Animals fed on a diet lacking foodstuffs containing this factor showed retardation of growth, greatly increased liability to infections, particularly of the respiratory and genito-urinary tracts, and susceptibility to a curious disorder of the eyes to which the names xerophthalmia or keratomalacia were given. Finally the classic researches of Mellanby provided evidence that the characteristic disturbance of calcification in rickets may arise as a result of a deficiency of a factor not unlike "fat-soluble A." Mellanby, however, was not quite convinced that only one factor was concerned. In one of his early reports he remarks, "Dealing only with fats, it is clear that the general similarity of distribution of the antirachitic and fat-soluble substances is striking, but, in detail, differences are evident which call for qualification and amplification." His caution was justified, for, in 1922, the work of McCollum, Simmonds, Becker and Shipley² showed that cod liver oil aerated at 100° C. for several hours retained much of its antirachitic action, but lost entirely its growth-promoting powers. Moreover, they drew attention to obvious inequalities of distribution of the two properties

in certain foodstuffs which supported the view that two separate factors were concerned. So was recognition given to the antirachitic vitamin D. A third member of the fat-soluble class, now labeled vitamin E, was discovered when Professor H. M. Evans at California and Professor B. Sure at the Arkansas Agricultural Experiment Station independently observed that the normal cycle of reproduction in the rat requires a dietary factor associated with certain fats, which from its distribution and properties is clearly distinct from any previously recognized vitamin.

VITAMIN A

Physiological Function.—Man and a wide variety of animal species may exhibit disordered health as a result of a deficiency of vitamin A. In general the train of symptoms is similar. Growth is retarded in the young, but this is, of course, not in any way remarkable, as almost every form of dietary deficiency will disturb the normal process of development to a greater or less extent. The characteristic symptoms are usually exhibited by the eyes. An early disturbance is chronic conjunctivitis often accompanied by the curious condition of *night blindness* or *functional hemeralopia*. The latter has been shown by Fridericia³ and by Tansley⁴ to be due immediately to a subnormal amount of visual purple in the retina and to a slow rate of regeneration of the pigment on passing from light to dark. It is this that makes the "night-blind" individual stumble when he goes from a lighted room into the half-light of dusk. The physicians of ancient Egypt and Greece recognized the condition and knew that it was promptly relieved by the administration of liver; a simple remedy still widely employed in parts of the world where the trouble is frequently encountered. Aykroyd⁵ has described how a wide variety of livers and liver oils are used as a popular and effective means for treating "night blindness" by the seafaring folk of the Newfoundland coast. It is not easy to judge how far one is justified in assuming from the relative scarcity of this condition in the towns of western Europe that the diet of the population taken as a whole is not deficient in vitamin A. One might naturally expect to learn that ophthalmologists frequently encountered "night-blind" patients, if, as animal experiments suggest, the disorder is one of the first manifestations of vitamin A deficiency.

The eyes may be the seat of another characteristic lesion ascribed to vitamin A deficiency. It is known as xerophthalmia because of the curious dry condition of the cornea and conjunctiva, a condition that frequently leads to ulceration and permanent damage. It is fully described by Bennett in his monograph on "*Oleum jecoris aselli*" (1841), but it is of interest to note that the clinicians preferred in those days to apply the oil directly to the affected eyes. The cures appear to have required some months, but as Mori showed in his full investigation of the disease (Hikan) as seen among children in Japan, they can be effected with surprising rapidity in uncomplicated cases by

giving the liver oil by the mouth.⁶ Xerophthalmia and hemeralopia as symptoms of vitamin A deficiency are common in many areas where the population is as a whole undernourished. The remarkable statement has been made by Wright⁷ that more permanent blindness of children in India is caused by these conditions than by ophthalmia neonatorum or trachoma. Treatment with cod liver oil is almost magical in its effect if given in time.

Xerosis of the cornea due to vitamin A deficiency does not seem to be a common disease in western Europe in normal times; but, as has been strikingly revealed by the investigations of Bloch⁸ in Denmark, it may appear with alarming suddenness if the diet is so altered as to reduce appreciably the amount of vitamin A present. During the early days of the War, Denmark sold the greater part of the butter she produced and her own population consumed in its place an increased amount of margarine. Within a year there was a sensational incidence of xerophthalmia, particularly in young children, associated, it is important to note, with greatly increased susceptibility to infection, in particular bronchopneumonia. "It is absolutely characteristic of these dystrophic children how little they are able to withstand infection, and how quickly they die of intercurrent fever." At the end of 1917 the export of butter was restricted by the government and almost immediately the number of cases of the eye disease fell to the pre-war level.

It is interesting to inquire what type of lesion is responsible for this abnormal state of the eyes. Before considering this, however, it would be well to bring under discussion the wider question of the structural changes which have been observed in the tissues of animals deprived of vitamin A. In 1928 Wolbach and Howe published the important results of their examination of such tissues. One characteristic change was found almost everywhere. It was the replacement of the various epithelia by stratified, squamous, keratinizing epithelium. Such keratinization had already been noted by Mori in the tissues of the eye, but no one had suspected that similar changes would be found widely distributed throughout the tissues. The morphological sequence showed that the replacement arose from a focal proliferation of cells arising from the original epithelium and not by differentiation or change of preexisting cells. The substitution of the epithelium was *not* secondary to infections. These observations are of the greatest importance, particularly since they have been confirmed by Goldblatt and Benischek,⁹ who thought it advisable to repeat the experiments, insuring that the animals did not suffer from a deficiency of vitamin D; the original experiments of Wolbach and Howe having been carried out before the differentiation of the two factors constituting the old "fat-soluble vitamin." There is some dispute as to the part infections may play in xerophthalmia but opinion is inclining to the view that they are not invariable accompaniments of the condition. Mouriquand, Rollet and Chaux describe observations of the rat eye in vitamin A deficiency by

means of the corneal microscope. From seven to nineteen days before lesions are visible to the naked eye, the cornea may show signs of transparency and thickening due to edematous infiltration. It is at this stage that infections may occur.

Frazier and Hu¹⁰ have recently described an outbreak of xerophthalmia in Chinese soldiers, some of whom showed cutaneous lesions with excessive epithelial keratinization leading to occlusion of the hair follicles and sweat glands. Both eye and skin symptoms were relieved by improving the food by the addition of milk, liver and cod liver oil.

Closely associated with vitamin A deficiency in experimental animals is an increased susceptibility to infection, particularly of the respiratory tract. How high the proportion of infectious disorders can be is shown by the percentage incidence in an experiment by Mellanby and Green¹¹ carried out on rats:

| | Per cent. |
|--|-----------|
| Xerophthalmia..... | 38 |
| Abscess at base of tongue..... | 72 |
| Lung infections..... | 9 |
| Infection of genito-urinary tract..... | 44 |
| Middle ear abscess and septic nasal sinus..... | 20 |

Indeed, so impressed were these workers with their findings and with the relative immunity of the animals receiving vitamin A that they tentatively proposed calling this dietary factor the "anti-infective vitamin." It began to look as if these facts might be brought into relation with the long recognized value of cod liver oil and butter in the treatment of phthisis and other chronic and intractable infective disorders on the one hand, and, on the other, with the structural changes in the epithelia which had been detected by Wolbach and Howe. Unfortunately, attractive as such correlation would be, it is far from being established. Investigation of a variety of serological and bactericidal tests has not yet provided convincing evidence that the defensive powers of the organism, at any rate as measured by these reactions, are reduced as a result of vitamin A deficiency. All who work with animals in vitamin research know that deprivation of vitamin A renders them more liable to infection, and yet, after the effects of the deficiency have been noticed, it is seldom that one observes an improvement in the condition—excepting the eye lesions to which we have referred—on administration of the vitamin. The same conclusion has been derived from studies on man. Neither Wright and his colleagues nor Paton could find any support for the view that administration of vitamin A protects children from common colds and related catarrhal conditions. Similarly, Orenstein failed to detect in a study of 750 cases any beneficial influence on the course of pneumonia by vitamin A therapy.

It may be argued that many of the diets today are low in vitamin A and that a large proportion of our population is suffering from

chronic deficiency of this factor. If such were true, it might be reasonable to assume that tissue changes had occurred over a considerable period, and that the damage would not be made good by a relatively short period of vitamin therapy. This is a plausible suggestion but against it we have to range two important pieces of evidence. To one we have already alluded, namely, that "night blindness" and xerophthalmia are relatively rare conditions in our large towns. If the population were in the borderland of vitamin A starvation one would expect these early symptoms of deficiency to be far more common. The second point is that the stores of vitamin A in the liver of the "man-in-the-street" are by no means negligible. Estimations of the vitamin in the liver of a large number of healthy and diseased individuals have shown that the reserves in this organ are often as large in cases which have died from chronic infection as in apparently healthy people dying as a result of accidents.

It is interesting to mention that much the same conclusions have been recorded by Wilson (for cases in Egypt), by Wolff (for cases in the Netherlands) and by Moore (for cases in Great Britain). The investigations of this last-mentioned worker indicate that normally the reserves of vitamin A in the liver at birth are relatively small. He has also provided more precise information from experiments with rats which show that, however well fed the female may be, the amount of vitamin A in the liver of the young at birth is not of a high order, as might have been expected. There is, however, clear evidence that accumulation of the vitamin in the liver tends to occur with age. Naturally, the extent to which the storage takes place depends on the character of the diet; and it is not without interest that diabetics frequently show exceptionally large reserves, doubtless attributable to their vegetarian diet. One might have expected to find in such cases a high resistance to bacterial infection unless one assumes that in diabetes there is impairment of the ability to mobilize the vitamin from the liver. Actually, however, it is well known that diabetics are unusually prone to contract infections.

Both Wolff and Moore estimate from their examination of human livers that something of the order of 16 or 24 per cent of the population shows some evidence of vitamin A deficiency. The direct attack on the problem of the relation of vitamin A to bacterial infection has not progressed very satisfactorily. It is unfortunately true that much of the work that has been reported must be rejected on the grounds either that the experimental conditions were unsatisfactory or that the interpretation of the findings can be criticized. One of the best investigations was that reported by Lassen who studied the progress of experimental parathyroid in rats after infection by the mouth or by subcutaneous or intravenous injection. The disease appears to have taken a more severe course in the animals that were deprived of vitamin A. On the other hand Topley, Wilson and Greenwood failed to find an increased susceptibility to mouse typhoid in rats which had

been fed on a diet deficient in vitamin A and which were then placed in contact with an infected colony. It is necessary to bear in mind, however, that the animals investigated by Lassen had been subjected to a much greater degree of deprivation prior to infection than those which Topley, Wilson and Greenwood studied.

The rôle of the reticulo-endothelial tissues, which are known to play an important part in some of the chemical processes which concern vitamin A in the body, is also believed to be intimately concerned with the production of antibodies and with other parts of the mechanism by which the body defends itself against invasion. It is possible that investigation of these tissues will provide more precise information, serving to clarify the position and to establish with more certainty how vitamin A assists in protecting the organism against infection.

Chemical Nature of Vitamin A.—The earliest investigations of the chemical nature of vitamin A were made on cod liver oil, which was at that time regarded as the richest source available. By saponification the active substance could be concentrated in the small fraction, rather less than 1 per cent, which can be extracted from the aqueous solution of soaps by fat solvents. This "unsaponifiable matter" proved on examination, however, to be a complex mixture of substances, the physical and chemical properties of which were such as to make a separation of the constituents a matter of the greatest difficulty. These studies failed to effect an isolation of the vitamin, but they provided important evidence which has since proved to be invaluable. In the first place, the behavior of vitamin A-rich concentrates from cod liver oil when treated with appropriate reagents led us to believe that the vitamin might reasonably be an unsaturated alcohol.¹² Secondly, by following up the clues given by a long-abandoned color reaction for cod liver oil, improved tests were devised which seemed to offer a certain means of recognition of the presence of the vitamin and possibly the basis of a method of estimation. Of these, the brilliant blue color with arsenic trichloride was the most striking and the most sensitive, but on the grounds of both convenience and safety this reagent has been displaced by antimony trichloride.¹³ Finally, evidence was obtained that selective absorption with a maximum at 3280 Å shown by fish liver oils in the ultraviolet region can be correlated with their vitamin A content.

Armed with these relatively simple tests, efforts were made to find richer sources of A than cod liver oil; there being reasons for believing that the most potent concentrates which had been made from that source contained probably less than 1 per cent of the vitamin. Fortunately, the search proved remarkably successful. After a period when it was thought that mammalian livers might be a valuable raw material, attention was abruptly turned once again to fish by the discovery that certain species may yield liver oils several hundred times richer in A than the usual run of cod.

Of particular interest was the halibut. Employing selected oils of high vitamin A value, as tested by the color reactions and the spectrographic measurements, the unsaponifiable matter was prepared. This, after preliminary treatment to remove a considerable amount of sterol and other inactive material, was subjected to fractionation in a molecular still¹⁴ at the very low pressure of 0.0001 mm. In previous experiments it had been found that at pressures of 0.001 mm. the vitamin was volatile, but that considerable inactivation occurred, possibly as a result of cyclization. At the much lower pressures attained in the very efficient form of molecular still, the distillation proceeds smoothly and the vitamin passes over without appreciable loss at a temperature of 137°–139° C.

Experiments almost identical in character were reported from Professor Karrer's laboratory at Zurich.¹⁵ The analyses and properties of the most active fractions separated corresponded satisfactorily with the view that the main constituent of the highly potent fractions was an unsaturated alcohol possessing the formula $C_{20}H_{30}O$. The material obtained is a clear, pale yellow, viscous oil which has not yet been converted into a well-characterized crystalline derivative suitable for effecting further purification.

In 1919 Professor Steenbock drew attention to a curious association which he and his colleagues had traced between the presence of vitamin A in plant foodstuffs and that of certain carotenoid pigments, in particular carotene itself.¹⁶ For example, it was shown that yellow corn (maize) is richer in A than the white variety and that the outer green leaves of the lettuce contain many times more carotene and vitamin A than the pale inner leaves. For a time, the more the association was investigated the more convincing did it appear, so long as plant products were examined. The correlation could not be supported, however, when animal foods were considered. One example was all that was needed to wreck the general application of the theory that vitamin A is itself a carotenoid pigment or a substance invariably associated with those coloring matters; cod liver oils could be obtained which, while good sources of the vitamin, were almost devoid of color. Recognizing this fact, Steenbock suggested that the vitamin might in such cases be present as the "leuco compound" of a lipochrome. We know today how near he then came to making a discovery of the first order; a discovery which in fact was not announced until ten more years had passed.

Following the clear line of experimentation which his arguments directed, Steenbock prepared crystalline carotene and administered it to rats whose growth had been inhibited by deprivation of vitamin A. The response was unconvincing. There were indications that the pigment was capable of restoring growth, but beyond reporting that positive results had been obtained, Steenbock and his collaborators did not make a definite claim; at any rate, not with the assurance derived from an experience wholly convincing. The reason is perfectly

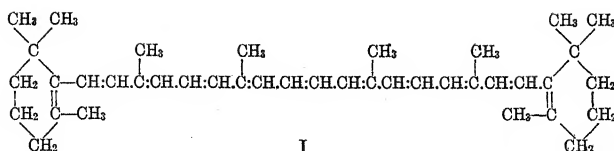
clear today. At that time vitamins A and D had not been differentiated. Steenbock suspected that carotene might replace "fat-soluble A" in the diet. When he put the idea to a direct test he was employing animals whose reserves of vitamin D were depleted to an unknown extent. This explains not only his own results, which one suspects were conflicting, but the entirely negative findings recorded by others who administered purified carotene as a source of A. Gradually the view was accepted that the association he had traced between lipochromes and vitamin in many foods was purely fortuitous and the subject was dismissed. Nearly ten years later, vitamin D having been discovered in the meantime, it occurred to Professor von Euler of Stockholm to repeat the crucial experiment of Steenbock. Insuring that the growth of his animals would not be prevented by the "limiting factor" of D deficiency, von Euler administered carotene and immediately obtained convincing proof that it possessed remarkable growth-promoting power.¹⁷ A short period of uncertainty followed, during which the question was raised whether his carotene was pure or whether the growth response might have been due to a highly active contaminant of the pigment, but final proof that carotene itself could replace vitamin A was not long delayed.

Now again rose the difficulty which was partly responsible for the rejection of Steenbock's views in 1920. Is carotene identifiable with vitamin A and, if not, what is its relation to the substance present in materials such as cod liver oil which may be very slightly colored? Moore at Cambridge has answered the question adequately. Carotene on passing into the tissues may be converted into a colorless, or almost colorless substance which appears to be the vitamin A of our earlier interests.¹⁸ He has summarized the relation in the following concise manner:

| <i>Carotene</i> | <i>Vitamin A</i> |
|--|--|
| Synthesized in plant. | Stored in animal. |
| Orange red in color. | Almost colorless. |
| No selective absorption in vicinity of 3280 Å. | Marked absorption at 3280 Å. |
| Greenish-blue color with antimony trichloride, with maximum at 5900 Å. | Vivid blue color with antimony trichloride, showing maxima at 5270 and 6060 Å. |

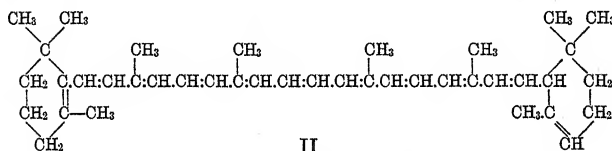
Let us consider the nature of the change involved. The constitution of the hydrocarbon carotene must be our first care; and it will also be necessary to refer in passing to that of a number of related lipochromes.

The brilliant series of researches by Karrer, by Kuhn, by Pummerer and others had revealed that the properties and behavior of the hydrocarbon carotene $C_{40}H_{56}$ can be satisfactorily represented by the symmetrical formula I. As will be seen, such a constitution represents



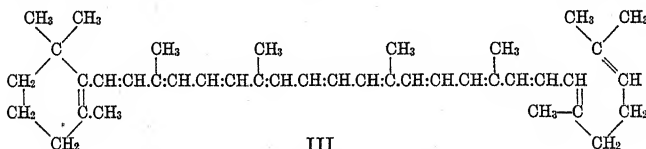
I
β-carotene $C_{40}H_{56}$. (Forms vitamin A.)

a compound which is optically inactive, but in Kuhn's laboratory, it was observed that certain samples of carotene may show optical rotation; moreover, it was found that by suitable methods the activity of preparations could be increased. By repeated crystallizations, by the employment of suitable adsorbents, and by chemical means, two distinct isomeric forms of the hydrocarbon were separated. They were labelled α - and β -carotene, respectively, and whereas the former was powerfully dextrorotatory, the second modification was optically inactive. Now it was necessary to elaborate a modification of the constitutional formula which would permit of optical activity. Not entirely convincingly at first, formula II was proposed to represent α -carotene;



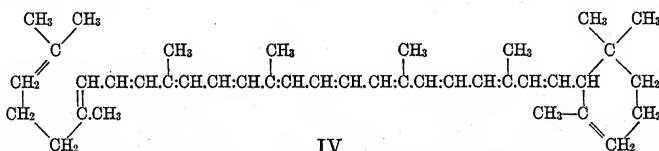
II
 α -carotene $C_{40}H_{56}$. (Forms vitamin A. 1 mol. only?)

formula I being retained for the β -form. More recently Kuhn and Brockmann¹⁹ have described a third form of the pigment, designated γ -carotene, to which the formula III is provisionally ascribed. The



III
 γ -carotene $C_{40}H_{56}$ (provisional formula).
(Forms vitamin A. 1 mol. only?)

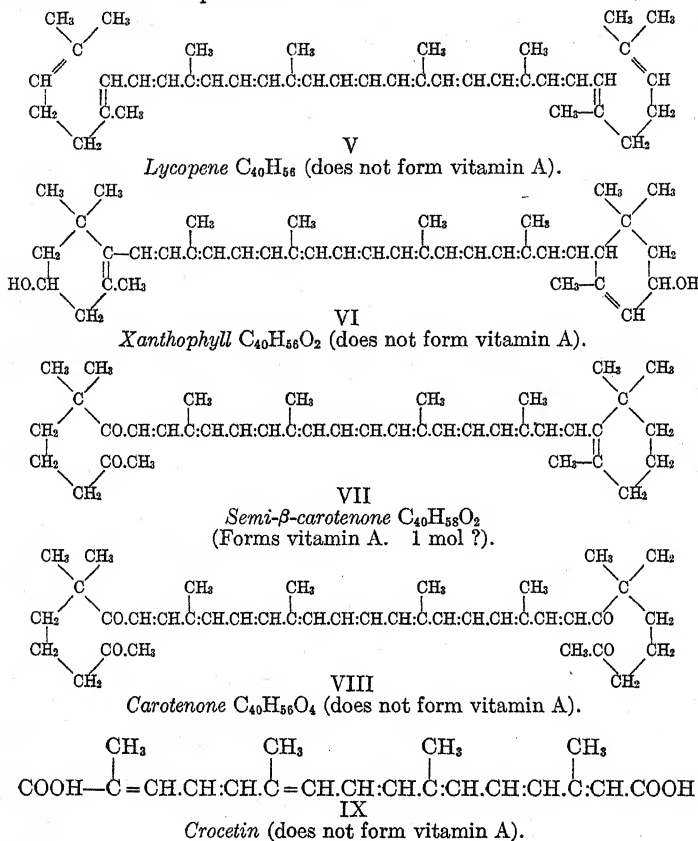
relation of these constitutions to those of the natural isomer from tomatoes, lycopene (V) and the artificially prepared isocarotene IV is



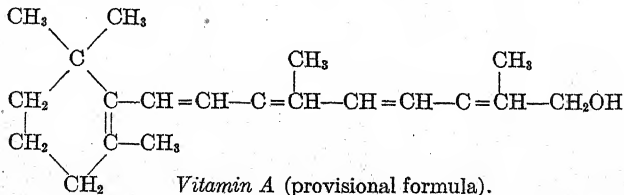
IV
Isocarotene $C_{40}H_{56}$ (does not form vitamin A).

clearly apparent. There are also given the structural formulae of

certain other natural lipochromes and one or two important derivatives to which it is important to refer.



The results of the studies which Professor Karrer and his colleagues have made of the properties of vitamin A as isolated from halibut liver oil concentrates are in the main fully confirmed by the observations made in Professor Heilbron's laboratory and at University College, London. The compound $C_{20}H_{30}O$ could be adequately represented by the formula



which would represent the product formed by rupture and hydration of the central ethylenic bond of the β -carotene molecule. But, it will

be observed, neither the α -carotene nor the γ -carotene molecule can split symmetrically in this fashion; their formulae permit of only one half of the molecule forming vitamin A. Lycopene and isocarotene containing no β -ionone ring will on the basis of these formulae be unable to yield the vitamin.

The biological evidence seems to fit these structural deductions tolerably well. Lycopene we know to be valueless to the growing animal—at any rate all species as yet investigated—so presumably the tissues have not the power to close the terminal carbon groupings and form a β -ionone ring. If this argument is sound, we might assume that α -carotene would possess only half the growth-promoting power shown by β -carotene. It has actually been claimed that biological tests support this view. The growth-promoting effect of β -carotene is stated to be appreciably twice that of the α - and γ -isomers, while in the former case the reserves of vitamin A stored in the liver are also greater.²⁰

It is not without importance that certain derivatives of carotene prepared by chemical means in the laboratory possess a biological activity which is in keeping with this suggested relation of structures. Thus, for example, the semi- β -carotenone VII is growth-promoting to an extent comparable with α -carotene (*i. e.*, one mol of vitamin A from half the pigment molecule)²¹ whereas isocarotene, the structure of which is probably related closely to the open-chain lycopene structure, is of no value as a source of the vitamin.²²

The xanthophylls and the pigments of the crocetin class are probably not convertible into vitamin A by animals, although from time to time several claims to the contrary have been made. The statement that xanthophyll serves as a "provitamin A" for fowls may need careful investigation in view of the curious preponderance of this pigment over carotene in egg yolk.

The physiological aspect of the change by which carotene is converted into vitamin A in the animal body must now be considered. Shortly after the discovery had been made of the relation between the two substances, it was announced by Olcott and McCann that it was possible to effect the conversion *in vitro* by incubating colloidal suspensions of the pigment in the presence of preparations of liver tissue. It should be remarked that interest had been focused on this organ by reason of the fact that most of the vitamin found in the body after administration of carotene is in the liver. Unfortunately, no confirmation of the rapid action of a liver "carotenase" has been forthcoming, in spite of many repetitions of the experiment in other laboratories. A series of investigations in the biochemical laboratories at University College has served to throw some light on the change as it occurs in the intact animal. When carotene in colloidal suspension in aqueous media is injected intravenously, whether into the portal vessels or the systemic circulation, it is removed with astonishing rapidity from the blood streams. The greater part is taken up by the Kupffer cells of the liver, in which the granules of pigment can actually be seen when

sections are examined immediately after the injections are given. Other organs well equipped with reticulo-endothelial systems also take up the pigment, but not so readily, or perhaps not so rapidly, as the liver. The liver with its load of ingested carotene does not appear to form vitamin A if it is immediately excised from the body and incubated for a reasonable period. What is more important, for incubation experiments of this type are at the best grossly unphysiological, is the observation that the conversion in the intact animal is relatively slow and, we think, inefficient. In cats it has been found difficult to show any formation of vitamin A from injected carotene; possibly the carnivorous animal is not equipped to deal with what is essentially a plant pigment, but in the rabbit, conversion can be distinctly demonstrated after some few days. The studies have brought up the possibility that carotene may not always be converted into vitamin A in the animal organism. It is known from recent studies at the Research Station of Imperial Chemical Industries at Jealott's Hill that the degree of conversion of carotene into vitamin A differs in different breeds of cows. It has long been known that on one and the same ration a cow of the Guernsey breed yields a milk of much deeper yellow tint than that given by a Shorthorn or Friesian. What has emerged from the researches to which I refer is the fact that the breeds giving the paler milks secrete relatively more vitamin A than those which give a rich yellow milk. In other words, the ratio of carotene to vitamin A, indicating the extent of conversion, differs considerably between different breeds, although regarded from the standpoint of nutritive value the milks may be equivalent, since the totals of pigment and vitamin A are approximately the same.

There are many problems concerning the storage of vitamin A in the liver which are of the greatest interest. One which has attracted considerable attention is the origin of the relatively large reserves which are found in the liver of certain fish. An interesting problem when we are focusing our attention on the cod, it becomes even more enthralling when we turn to consider halibut livers which may contain 20 per cent of an oil containing as much as 15 per cent of the vitamin. The difficulty is that unless one assumes that these fish possess the power to synthesize vitamin A, it is almost impossible, at first, to believe that the stores can possibly be derived from food which is known to be curiously deficient in this respect. If we reject the idea of synthesis, and there is at the moment no justification for our seriously entertaining it, we can toy with the idea that although the actual concentration of vitamin in the food may be small, the amount eaten is very large and gradual accumulation in the liver would in time account for the large reserves actually observed. Some evidence supports this view, certainly in so far as the idea of accumulation is concerned; Macpherson having found that the older the Newfoundland cod is the larger tends to be the amount of A stored in the liver. But there are interesting developments in another direction. The ultimate

source of food for all marine animal life is the phytoplankton. So far as has been determined the green diatoms contain carotene and no other substance known to be intimately related to vitamin A. The next stage in the cycle of life in the sea is the vast, mixed population referred to collectively as zooplankton. Foremost in representing this class, at any rate so far as our subject is concerned, are the copepods and minute crustacea. They contain little or no vitamin A, if one relies on the usual chemical and physical tests or if one judges from biological experiments with rats.

They do, however, contain pigments which have recently received some attention and which are in all probability very widely distributed in marine animal life. These pigments are responsible for the delicate pink color of salmon flesh, and extracts containing them do not promote growth in rats deprived of vitamin A. In a recent study evidence has been obtained which seems to indicate that in the developing eggs of a fish such as trout these pigments, derived originally we think from the carotene of diatoms, are to some extent converted into vitamin A. It is by no means beyond the bounds of possibility that the intermediates between carotene and vitamin A differ in different species, and that in fish one stage is represented by pigments which are without biological value for rats.²³

Vitamin A Content of Foodstuffs.—Such investigations as have been made have failed to detect the presence of vitamin A in any plant foodstuffs. The assumption appears justified that the growth-promoting activity of these materials as demonstrated by animal tests is to be ascribed to their content of carotene. This renders it a comparatively simple matter to make estimations of the vitamin A potency of foodstuffs of plant origin because the accurate determination of carotene by colorimetric or spectrometric methods is a matter of comparative simplicity. Small amounts of carotene are usually present in seeds, but they are insufficient to make the ordinary cereal foods, except yellow maize (corn), of any significant value as sources of this vitamin. The appearance of carotene in plants in considerable quantities is associated with the growth of the vegetative system and with the development of the chlorophyll mechanism in relation to light and assimilative activity. As a rough guide, the amount of chlorophyll in green plants may be taken as indicating the amount of carotene present. This explains why the outer green leaves of cabbage and lettuce are much more valuable as sources of vitamin A than the inner etiolated leaves. Storage of carotene may occur in fruits, roots and tubers, but apart from the carrot, the chief plant foods of value in the diet as sources of this vitamin are the green vegetables.

When we come to the animal foods, we find a close relationship existing between their value as sources of vitamin A and the diet on which the animal has been reared. On a diet containing little vitamin A there is a tendency for the reserves of the animal to become exhausted, and in such a case the tissues of the animal will be poor as

sources of the vitamin. For the same reason, secondary animal foods such as milk and butter possess a low value when derived from animals on a ration showing this type of deficiency. Animal foodstuffs may contain both carotene and vitamin A. As already pointed out, the proportion of these is influenced in the first place by the diet, while important variations are also introduced by the extent to which the tissues of the animal concerned convert the pigment into the vitamin. If we again take the example of milk, we find on the one hand that the Jersey and Guernsey cows tend to produce a milk of high carotene content with relatively little vitamin A, whereas the goat or the buffalo converts practically all the absorbed carotene into vitamin and secretes a milk almost devoid of yellow pigment.

Both carotene and vitamin A in foodstuffs are relatively stable to the usual processes by which food is preserved or cooked. They are both susceptible to oxidation and this is the most common cause of any depreciation which may occur. The old-fashioned methods of drying food frequently brought about considerable destruction of both substances, but recently processes have been introduced by which it is possible to remove the water without any undesirable secondary changes occurring. Thus, for example, whereas sun-dried hay is usually of negligible vitamin A content, it is now possible to dry grass rapidly so as to obtain a nutritious and palatable foodstuff suitable for supplying carotene to animals during the winter at a time when there is a tendency for their basal ration to be deficient.

VITAMIN D

Physiological Action.—To a generation that sees rickets fast disappearing as a result of suitable dietary treatment, it must seem incredible that ten years ago this scourge afflicted to a greater or less extent nearly 80 per cent of the children in the large towns of western Europe and America. Moreover, it must be difficult to comprehend that centuries of clinical experience had failed to produce a clear-cut theory regarding the relation of the disorder to defective diet. It is true that scattered everywhere through the extensive literature on rickets one finds countless theories ascribing the defective calcification to nutritional disturbances, but on the numerical basis alone these opinions are discountable by the equally weighty statements that the disease is caused by defective hygienic surroundings, hereditary influences and other causes.

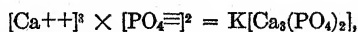
The brilliant series of researches carried out by Professor E. Mellanby brought an immediate clarification of the position to those who had eyes to see and ears to hear. His experiments on dogs admitted no possible doubt that dietary factors were fundamentally important in insuring normal disposition of lime salts in developing bone. It is true that his work did not immediately dispel the conflict between the adherents of the two main groups of theories regarding the origin of rickets, for the supporters of the view that lack of fresh air and sun-

light were more important factors than faulty diet produced evidence almost as convincing as that provided by the clear-cut experimental results of Mellanby. It is now almost commonplace knowledge how the reconciliation between these conflicting schools of thought was brought about. The beneficial influence of sunlight or ultraviolet radiation in preventing or curing rickets was established by the clinical work of Palm, Huldshinsky and Hess and Unger,²⁴ and it was not long before ample evidence from laboratory experimentation provided support for their observations on young children.

Then came the classical work of Professor Steenbock and his colleague, Dr. Black, demonstrating the remarkable fact that food mixtures which will lead to the production of rickets in young animals can be endowed with protective properties if they are exposed to the ultraviolet region of the spectrum.²⁵ The photochemical change which occurs when foodstuffs are thus irradiated was quickly found to concern a constituent of the fatty part of the ration. The progress of the search for this constituent is one of the most enthralling romances of modern biochemical investigation. It led from the fats to the unsaponifiable matter, from this fraction to the sterol it contained and from the main constituent of the sterol fraction, namely, cholesterol, to an almost insignificant amount of a related sterol, ergosterol, which frequently is found in close association. This interesting substance, previously of the slightest academic interest, having originally been isolated as a constituent of ergot, suddenly sprang into prominence as the parent substance of one of the most important dietary principles.

As will be described later, ergosterol undergoes photochemical change when it is exposed to suitable radiation in the ultraviolet spectrum. This change is, so far as we are aware, due to a molecular rearrangement giving rise to one of many possible forms of this sterol. It is a remarkable example of the relation between the biological action of a substance and its chemical constitution that whereas one form of this molecule plays an essential rôle in determining the balance of calcium and phosphate in the body fluids, others, including the naturally occurring form and a variety of isomers which have been made artificially, possess no such action.

The physiological function of vitamin D has been extensively investigated during the past few years. The fundamental fact to bear in mind is that the deposition of mineral salts in the developing bony structures is determined by precisely the same physicochemical laws as those which lead to the separation of the lime salts from simple aqueous solutions. Perhaps the simplest illustration is to take the case of tricalcium phosphate $(\text{Ca}_3(\text{PO}_4)_2)$ separating from simple aqueous solutions. The concentration of free calcium and free phosphate ions determine whether or not solid calcium phosphate separates. If the product of these concentrations exceeds the *solubility product* as represented by the equation



solid calcium phosphate must be deposited. In any system where the concentration of these two ions is such that the *solubility product* is not reached, calcium phosphate cannot separate. By raising the concentration of one ion or of both to the extent required to exceed this value, the solid calcium phosphate will immediately separate from the solution. If the blood and the tissue fluids of young animals contained sufficient of these ions in the free condition to bring about separation of the salt, it would be generalized and would not be confined to the developing bone. It is clear, therefore, that normally these fluids must contain less than the appropriate concentrations of the ions. Actual analyses of blood show that under normal conditions this is true, for, although the total amounts of calcium and phosphoric acid may be larger than those required to give the necessary concentration were all present as free ions, a considerable proportion in each case is known to be represented by compounds which are not in this respect ionized.

A recent examination of this interesting phenomenon, which has been extensively studied in the past, is recorded by Benjamin and Hess. In normal serum they find that of the calcium which is in a form capable of passing a diffusion membrane, two thirds is an adsorbable complex of calcium and phosphorus and only one third in the ionized state. About three quarters of the nondiffusible calcium is bound in an unknown manner to the serum proteins. Of compounds of phosphoric acid there is a wide range, none of which contributes significantly to the amount of free phosphate ion in the blood. It is clear, therefore, that localized calcification exhibited by the young developing animal must be conditioned by a purely local mechanism.

We owe it to the brilliant series of researches of Dr. Robison and his colleagues that the chief facts concerning the nature of this mechanism have been discovered. He has shown that at centers of calcification and, it is of interest to note, at other sites where alteration in the balance or equilibria affecting calcium and phosphate is necessary, there is found an enzyme possessing the power of breaking down some of the organic esters of phosphoric acid normally present in the blood stream. By this activity a local increase in the concentration of free phosphate ions is produced in the immediate vicinity of the calcifying cartilage which is usually sufficient to cause the "solubility product" to be exceeded.

It is not always, however, that this mechanism is effective in causing deposition of insoluble lime salts. In rickets an examination of the blood will reveal that the concentrations of free phosphate and sometimes of calcium are markedly below the normal level. In such cases the activity of the bone enzyme, phosphatase, may be insufficient to raise the local concentration of phosphate ions to the required level, so that calcification does not take place. Rickets is essentially a disordered condition of the blood and most of the modern authorities accept the view that in many respects it can be viewed principally as a phosphate deficiency.

It is obvious that the biochemical picture is by no means as simple as has been described here. In so complex a medium as blood, many other factors must exert an influence on the deposition of bone salts. The influence of the bicarbonate system in the blood is revealed by the calcium carbonate constantly associated with the main bone salt, hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Moreover, the problem is rendered more complex by the curious and little understood part played by certain of the blood proteins, which are known to form loose compounds with calcium. Our immediate interest, however, is to inquire how the administration of minute traces of vitamin D will with such remarkable celerity lead to the deposition of inorganic salts in the bone.

It is quite certain that in rickets there tends to be a condition of negative balance in respect to phosphate and calcium. In other words, although the diet may contain ample supplies both of lime and of phosphates these may be imperfectly assimilated, in spite of satisfactory absorption having occurred. Rather is it that the processes leading to retention are impaired and that by reexcretion a negative balance is brought about. In all probability the effect of the vitamin is to increase the retention. It has been suggested that this may occur by changes being brought about in the permeability of the gut to the salts of calcium, but as yet there is not satisfactory evidence from experimental investigations to justify one in regarding this as more than an interesting speculation.

One of the most striking features of the effect of vitamin D on the retention of calcium is the extent to which it can be produced. In the early days of investigation of irradiated ergosterol, it was found that the administration of large doses might be followed by the appearance of calcification in many other tissues than those where it normally should occur. Indeed, it was possible to obtain almost generalized calcification by administering to animals many thousand times the dose of the vitamin preparation necessary to induce normal bone formation. Not only were these results obtained in the laboratory, but, before it was generally appreciated that the response to vitamin D might be in this sense quantitative, indications were obtained that health might be endangered by overdosage. For a time it was thought that a toxic by-product of the irradiation process might be responsible for the effect and that the hypercalcification was a feature of the poisoning which could be attributed to this substance. The evidence gradually accumulated in the other direction, however, and ultimately when vitamin D was obtained in pure condition, the hypothesis had to be finally abandoned. It is now certain that the level of calcium and phosphate in the blood can be raised to extraordinary heights by suitable dosage of the vitamin itself.

Harris has recently reported that he and Robison have observed that the serum of rats showing such abnormally high concentrations of calcium or phosphate will induce remarkably heavy calcification of cartilage in sections of developing bone investigated *in vitro*.

The picture presented by animals suffering from excessive administration of vitamin D is superficially similar to that shown by animals which have received large doses of the parathyroid hormone. It is not surprising, therefore, that investigators have been tempted to raise the suggestion that vitamin D influences the level of calcium in the blood through the agency of the parathyroid glands. This view is supported with particular emphasis by Taylor and his colleagues at the University of Toronto, who claim to have obtained clear evidence that vitamin D does not evoke the characteristic response in animals from which these glands have been removed. The evidence for the moment is somewhat conflicting for Dale, Marble and Marks have failed to obtain full confirmation of the rather crucial experiments reported by the Toronto investigators. They found that dogs develop a high level of calcium in the blood on administration of vitamin D as readily after removal of the parathyroid glands as before the operation. The position is not quite clear and further work is awaited with great interest. It would be surprising if two unrelated agents independently produced a physiological condition so similar as those which appear after administration of excessive doses of vitamin D or after injection of large doses of parathyroid hormone.

An important physiological question which has not yet been fully answered is whether or not the vitamin D formed by the photochemical isomerization of ergosterol is identical with the antirachitic substance—or possibly there are more than one—found in such natural products as cod liver oil. Although many have done so, it should not be assumed that the synthetic or artificially produced compound is not modified in its passage through the animal tissues. On the other hand, there are indications from more than one direction that the vitamin formed by irradiation of ergosterol may actually be changed in structure after absorption into the animal body and that one or more of the derivatives may possess antirachitic powers but of an order differing from that of the original substance.

Chemistry of Vitamin D.—The photochemical changes which occur when ergosterol is exposed to ultraviolet light have been extensively investigated. They are by no means simple. Vitamin D itself shows selective absorption in the ultraviolet part of the spectrum, so that unless the irradiation is carefully controlled there will naturally be photochemical destruction of the vitamin D itself. The best conditions for irradiation are now reasonably well defined. Apart from the desirability of employing filtered radiations which reduce to some extent the intensity in the areas likely to cause secondary destruction of the vitamin or other undesirable changes, the choice of the solvent and the time of irradiation have to be decided after careful consideration. Thus, for example, Reesnik and van Wijk describe the irradiation of well-stirred oxygen-free solutions of ergosterol with light filtered so as to transmit the narrow region between 2890 and 3000 Å. It is particularly important to filter off the shorter wave and it is

stated that the best yields are given by the use of light from the magnesium spark which contains a large proportion between 2780-2900 Å, the shorter waves being filtered off by means of benzene or toluene. The material that is obtained after ergosterol has been irradiated until biological tests show that the optimum amount of vitamin D has been formed is a resinous gum of most unpromising character. It is not a difficult matter to remove the unchanged ergosterol, since this can be quantitatively precipitated by digitonin. The product remaining is one to which it is difficult to apply the ordinary simple methods of purification by fractional crystallization.

The first successful effort to isolate an active crystalline material was that made by a group of investigators at the National Institute for Medical Research in London.²⁶ They fractionally distilled, or perhaps it would be more correct to say sublimed, the ergosterol-free gum in the very high vacuua of the order of 0.0001 mm. By this means it was possible to obtain without the use of very high temperatures a series of fractions, from one of which a highly potent crystalline material was separated. It was given the name *calciferol*, and at first its discoverers were inclined to think that it was an antirachitic vitamin in reasonably pure condition, but they were faced by a difficulty in that the biological potency was of the same order as that of the parent material. This they hoped to overcome by expressing the opinion that "if the crystals are essentially a single antirachitic compound (with only unimportant impurities) there must exist not less than two substances possessing intense antirachitic activity."

About the same time Windaus described the isolation from the products of the treatment of irradiated ergosterol preparations with maleic anhydride of a crystalline material which he also at first believed to be a derivative of an antirachitic vitamin.²⁷

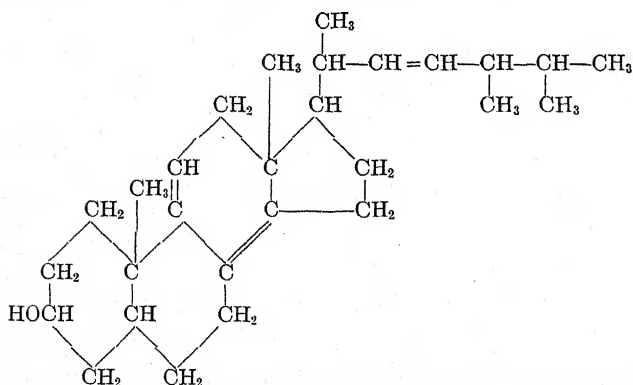
The *calciferol* of the English group of workers and the *vitamin D₁* of Windaus showed reasonably good agreement as regards properties except that the former showed a much higher optical rotation: $[\alpha]_{5461}^{20} + 260$ as compared with $[\alpha]_{5461}^{18} + 175$. This difference could not be satisfactorily explained, and it led to further investigations being made in the two laboratories. These soon revealed the fact that in each case the preparations which had been obtained were actually mixed crystals, complexes which members of the sterol family are very prone to form. The Medical Research Council team separated their preparation into two fractions, on the basis of the solubility of the derivatives formed by reaction with 3,5-dinitrobenzoyl chloride.²⁸ The substances regenerated from these derivatives were sharply differentiated. One was a beautifully crystalline compound showing greatly enhanced antirachitic properties, while the other was physiologically inert when purified. To the former the name *calciferol* was transferred, while the latter was termed *pyrocalciferol*, in view of the probability that it was a secondary product resulting from the heating during this sublimation process.

Windaus and his colleagues at Göttingen also applied the separation by means of 3,5-dinitrobenzoyl chloride to the product which they had previously obtained, and they were equally successful in effecting a separation of the active principle.²⁹ This material, which they provisionally termed *vitamin D₂*, is clearly identical with the new preparation of *calciferol*; and subsequent reinvestigations have served only to confirm the belief that these are pure preparations of vitamin D. It will be observed from the formulae which are given in the table that all four preparations have the same molecular formula as ergosterol.

PROPERTIES OF "VITAMIN D" PREPARATIONS

| | M.p. | $[\alpha]_{5461}^{20}$ EtOH | Maximum absorption | Biological potency | Formula |
|------------------------------|--------|-----------------------------|----------------------|--------------------|-----------------------------------|
| Calciferol I. | 123-5° | +260 | 2700 | 20,000 | C ₂₇ H ₄₂ O |
| Vitamin D ₁ | 124-7° | +175 | 2680 | 23,700 | C ₂₇ H ₄₂ O |
| Calciferol II. | 114-7° | +122.5 | 2650 | 40,480 | C ₂₇ H ₄₂ O |
| Vitamin D ₂ | 114-5° | +120 | 2630-2650 | 44,600 | C ₂₇ H ₄₂ O |
| Pyrocalciferol. | 93-5° | +624 | 2960 2840 2740 | 0 | C ₂₇ H ₄₂ O |

There is now no question that vitamin D is other than an isomeric form of the parent compound. It is impossible at this stage, seeing that we must still admit many serious gaps in our knowledge of the structure of ergosterol itself, to say in what respects the molecule becomes altered under the action of ultraviolet irradiation.

Ergosterol C₂₈H₄₄O (provisional formula).*

* It will be observed that the formula of ergosterol is here given as C₂₈H₄₄O in place of the long accepted C₂₇H₄₂O. Recent analyses and work on the constitution of this sterol are in better agreement with the C₂₈ molecule.

Several isomeric forms of ergosterol have been prepared synthetically, but none of these has any detectable antirachitic power.

Vitamin D in Foods.—There is no convincing evidence that vitamin D ever occurs in foodstuffs except directly or indirectly as a result of the exposure to light. This is not to say that the possibility of photochemical change is ruled out entirely, because from more than one direction suggestions have been prompted that the facts cannot always be adequately explained by assuming that the action of light on ergosterol is necessary. It is, for example, not entirely a simple matter to account satisfactorily for the very large stores of vitamin D which may be found in the livers of certain fish. Vitamin D might be formed from ergosterol in those species which inhabit depths to which the necessary light radiation can penetrate, but this seriously reduces the available sources. The surface plankton which might well be the starting point have, to a certain extent, been examined. The microscopic plants do not appear to contain any significant amounts of vitamin D. In this respect they resemble the majority of land plants; but the oils extracted from zooplankton may show a definite if rather low antirachitic value.

Similarly, many of the species intermediate between plankton and the larger fish may contain vitamin D, but the amounts are seldom more than traces. To account for the large stores of vitamin in such a material as halibut liver oil we must assume either that it represents long accumulation of the small contributions from the daily food supply, or, alternatively, that synthesis *de novo* occurs in the tissues.

The majority of plant foods are very poor sources of vitamin D. There is evidence that immediately after exposure to sunlight appreciable antirachitic powers are exhibited by green vegetables, but in the normal course of events this is not detectable by the time they have been picked, marketed and are ready for consumption. There is one group of plants, however, of very great importance in relation to vitamin D, namely the lower species represented by the yeasts and fungi. Many of these contain considerable amounts of ergosterol; it will be remembered that it was from ergot that this substance was first isolated. These organisms have assumed considerable importance as sources of ergosterol for the commercial manufacture of vitamin D; and it is of interest to note that the yield of sterol is considerably influenced by the species and by the cultural conditions under which it is grown. At the present time carefully selected strains of yeast are grown under appropriate conditions so as to provide the maximum yield of ergosterol.

Vitamin D in animal foods, if one rules out the possibility of synthesis in some species, arises either from the diet or from direct irradiation of the tissues. In the majority of cases the former is the more important factor determining the amount present. One of the best natural sources of vitamin D, if we except the fish liver oils, may be egg yolk; but unless the diet of the birds contains adequate vitamin

D or unless the birds are being exposed to sunlight or artificial irradiation, the yolks will be deficient. It is interesting to note that the liver oils of mammals are usually devoid of any but the merest traces of vitamin D. This is true even when the diet has been unusually rich in respect of this factor or when the animal has been exposed to a large amount of ultraviolet irradiation. This leads us to suspect that vitamin D may be readily broken down in the mammalian organism, and it is interesting to note that direct proof that such is a fact is being obtained.

The vitamin itself in the pure condition is relatively a stable substance. It is not destroyed by heating unless the conditions are such as to favor oxidative change, in which case inactivation occurs, but at a rate much lower than that exhibited by vitamin A. Foodstuffs which contain vitamin D can be regarded as unchanged by the usual preserving and cooking processes. This has been studied accurately in the case of milk, the vitamin D content of which is primarily dependent on that of the diet of the female. Normally, cow's milk has a rather low antirachitic value so far as the nutrition of infants is concerned, and an important practical development during the past few years has been the introduction of methods for enhancing the vitamin D content of milk by supplementing the diet of the cow either with natural foods, such as cod liver oil, or by means of artificial preparations, such as irradiated yeast. By either method, the original D value of the milk can be increased many times.

Another important contribution to the practical issue has been the discovery that the vitamin D content of milk can be effectively raised in a direct manner by very short exposure to ultraviolet radiation. This exposure must be carefully estimated so that the light does not induce oxidative changes adversely affecting both the nutritive value and the palatability of the fluid, but when suitable apparatus is employed there is no difficulty in obtaining the required result with a few seconds' treatment.

VITAMIN E

Physiological Function.—From time to time investigators had attempted the task of rearing young animals on artificial diets believed to supply all the recognized essential dietary constituents. Often growth was found to be satisfactory and general health excellent, but when the reproductive powers were examined it was found that the animals were either unable to breed or were incapable of rearing the young which might be born. In 1920 Mattill and Conklin reported that rats reared on whole cow's milk were usually sterile and that the failure to breed might be due to the lack of an undiscovered dietary essential. More precise information was provided when in 1922 Evans and Bishop⁸⁰ reported that young female rats fed on what were believed to be complete "purified diets" exhibited curious disturbances of the reproductive processes. The estrus cycle was in general un-

disturbed and no abnormalities were noted in the mating and implantation. Invariably, however, fetal death followed by resorption occurred about the twelfth day. The disorder was found to respond to treatment by dietary means, strong indications being obtained that a substance of the vitamin type associated with certain vegetable fats was the protective or curative substance. Independent studies of a very similar character caused Sure³¹ to reach a similar conclusion and thus the history of what was later to be known as vitamin E began.

At first there was some opposition to the view that a new vitamin had been discovered. Nelson and his colleagues, in particular, subjected the idea to searching experimental tests, the results of which for four years sustained their skepticism. It is not without interest to examine this controversy because it provides an excellent example of the unsuspected difficulties which beset work in the field of experimental nutrition. Nelson and his coinvestigators had observed that lard—which formed one of the main constituents of the food mixture employed by Evans and Bishop—brought about sterility when added to diets composed largely of whole milk powder, which in themselves permitted reproduction. Not unnaturally, they thought that the failure to breed exhibited by the rats of the Californian workers was due to a disturbance of food and calorie intake caused by the increased proportion of fat in the form of lard. The work of Mattill, Carman and Clayton and of Evans showed, however, that this interpretation would not serve, as high proportion of other fats such as butter did not induce sterility. The explanation proved finally to be—and few would have entertained the idea before the experimental results were available—that lard possesses the curious power of causing inactivation of vitamin E.

The abnormal condition leading to the death and resorption of the fetus has been closely examined by Evans and his coworkers.³² Defects in the allantois and yolk sac are suspected of being in the main responsible. In the male animals serious damage to the testis may result from E deficiency and lead to a permanent sterility.³³ Evans suspects that a considerable degree of infertility is apparent before the structural damage to the testicular tissue is marked.

The actual function of E in the body is unknown. The apparently unrelated effects on the male and female reproductive tissues and, moreover, the suspicion that this complement in the diet may also be essential for lactation, have from time to time led to the suggestion that more than one factor is concerned. Until a year or so ago, the idea of a unity was in favor, but quite recently arguments in favor of multiplicity are again in evidence.

The important question of the relation of vitamin E to the numerous hormones which regulate the normal reproductive cycle has not yet been adequately examined. In particular is it of importance to explore more fully the possibility that the termination of pregnancy which characterizes E deficiency may be associated with disturbed

function of the anterior lobe of the pituitary or of the corpus luteum. Evans, however, is disinclined to support the relation to the anterior lobe, having found that parenteral administration of the fresh hypophyseal substance does not enable the animal deprived of E to carry through a normal gestation.

Only recently have inquiries been made to determine whether E deficiency is of everyday practical importance and already interesting observations are being recorded, although as yet they are inadequate for us to form an idea of the extent to which E deficiency occurs in men and women and in the important farm animals. Vogt-Möller and Tutt have both described the successful treatment of sterility in cows with wheat germ oil, while the former authority writes hopefully of the results of similar treatment of sterility of unknown origin in women.³⁴ Developments along these lines are to be awaited with great interest.

Chemistry of Vitamin E.—The chemical nature of this vitamin was studied by Evans and Burr some years ago and until recently curiously little had been done since their original reports were issued.³⁵ The best raw material was found to be the oil derived from wheat embryo, although the potency may vary considerably with the sample. The vitamin can be concentrated without difficulty in the unsaponifiable fraction and by removal of the sterol this becomes further enriched. Attempts were made to isolate the vitamin from the residue by the usual procedures of crystallization, by the use of selective solvents and by distillation at low pressures. These, although to a large extent unsuccessful so far as the main object was concerned, served to reveal the active substance as being curiously stable. The properties which have been described, stability to hydrogenation, loss of activity on bromination and acetylation, etc., are such as might be expected if the vitamin were an unsaturated alcohol related to the sterols or of the type to which vitamin A belongs.

The great success which has followed the application of spectroscopic methods of examination in the study of vitamins A and D has prompted a number of efforts to trace relations between the absorption spectra and biological activity of materials and concentrates containing vitamin E.

Reexamination of the constituents of the nonsaponifiable fraction of wheat germ oil has shown that biological activity seems definitely to be associated with a substance showing a broad absorption band with a maximum at 2940 Å and exhibiting no signs of fine structure.

The nature of the substance responsible for this band has not yet been determined. It is an unsaturated compound which can be readily reduced by hydrogen in the presence of palladium without substantially affecting the intensity of absorption. This is in agreement with the previous observation that the vitamin is stable to hydrogenation. On acetylation the characteristic absorption band disappears but is again shown by the material regenerated after hydrolysis.

The properties of this compound are such as to suggest a cyclic

ketone capable of exhibiting keto-enol changes. It will be recalled that it is stated that the biological activity of vitamin E fractions is lost on acetylation.

WATER-SOLUBLE VITAMINS

Little more than eight years ago we were happy in recognizing but two accessory dietary factors in this group, water-soluble B (also known as the antineuritic or antiberiberi vitamin) and the antiscorbutic vitamin C. Such a simple state of affairs was too good to last, and any complacency we may then have shown has been rudely dispelled by the bewildering rapidity with which the position has been rendered exceedingly complex by the discovery of new "water-soluble" factors. Vitamin C has throughout retained its individuality; and to our general relief it is now established beyond all further doubt by the isolation, proof of constitution and, we think, by the final step of synthesis. "Vitamin B" by contrast forms a depressing subject for discussion. The first suspicions that "vitamin B" might be a complex dietary factor arose from the observations that whereas some preparations of the vitamin would cure "experimental beriberi" in animals and at the same time restore growth or lost weight, others might exhibit only one of these activities. The position was not really clarified until the investigations of Smith and Hendrick³⁶ were reported. They found that Seidell's preparation of "vitamin B" would, in very small doses, cure the so-called "polyneuritis" of pigeons, but that it was quite inadequate to promote growth in young rats fed on the appropriate deficient diet.

When, however, the Seidell concentrate was supplemented with a small amount of yeast which had been autoclaved to destroy its "antipolyneuritic" potency the young rats immediately resumed growing. The two factors were then accepted as distinct and were given separate labels. In England they were termed B_1 and B_2 respectively; in the United States they were known as F and G. In such manner have the various biological properties of "vitamin B" been investigated with ever-increasing precision. No less than five, or possibly six, factors have thereby been differentiated, but it would be impossible in a small space adequately to describe the steps by which their individuality has been established. For convenience an attempt has been made to summarize the complex position that exists at the present time in the following manner:

Vitamin B₁ (Vitamin F), the "antineuritic vitamin" of the early workers on beriberi. Also known as *torulin* (Peters). Required by animals for growth. Deficiency leads to disorder of the nervous system.

Vitamin B₂ (Vitamin G), described by Goldberger as the *antipellagra* vitamin. Also known as the *antidermatitis* factor. Required for growth. Deficiency may lead to appearance of skin lesions superficially resembling those seen in pellagra.

Vitamin B₃, described by Williams and Waterman³⁷ as necessary

for growth of pigeons. Probably not needed by the rat. Needs of other animals not known.

Vitamin B₄, described by Reader³⁸ as necessary for growth and normal health of rats. First labelled B₃ but this now refers to the factor above.

Vitamin B₅, described by Carter, Kinnorsley and Peters⁴⁰ as necessary for growth of rats. Differentiation from other factors not quite sharp.

Vitamin B₆, the factor Y described by Chick and Copping.³⁹ Differentiation not yet quite satisfactory.

Of these five or six factors, grouped conveniently but possibly unwisely under the title of "vitamin B complex," only three are sufficiently clearly recognized to merit a full discussion. These are vitamin B₁ (F), vitamin B₂ (G) and vitamin B₄. They will now be considered separately.

VITAMIN B₁ (VITAMIN F)

Physiological Function.—In this dietary essential we recognize the old-timer which emerged in the days when the etiology of beriberi was engaging the attention of three brilliant groups of pioneers thirty years ago. Eijkman, Grijns and Vodermann in the Dutch Indies; Frazer, Stanton and Braddon in the Federated Malay States; Veder and his colleagues in the Philippines. Their work pointed indubitably to the existence in rice polishings of a substance which protected the organism from the development of lesions responsible for the characteristic symptoms of "beriberi." What are the characteristic lesions of this disease? In considering this question we must constantly bear in mind that it is not often that uncomplicated deficiency disorders are encountered in man. An examination of human dietaries will often show that the individual consuming them is being subjected to more than one deficiency. It is true that one of these may be the major defect of the diet and that the influence of this particular inadequacy may be the first to be detected, but it is difficult in many cases to disentangle satisfactorily the complicating factors and to obtain a clear picture of the nutritional disorder which it is desired to study. Thus, "beriberi" as seen in the populations of the East is often a highly complex clinical picture and one which may fail to respond to simple "vitamin B" treatment in the sharp manner in which the convulsions of the "polyneuritic" pigeon disappear when the same therapeutic measures are applied.

This statement particularly concerns the disease as seen in children or adults. Infants at the breast may show a true picture of vitamin B₁ deficiency if they are being nursed by deficient mothers and this condition of "infantile beriberi" is reasonably comparable with the conditions producible in laboratory animals by carefully regulated feeding. The discovery by Eijkman that a disease resembling beriberi could be produced in birds by feeding them on polished rice was an

important step forward because it provided a simple method for extending investigations not only of the etiology of the disorder but of the nature of the curative substance.

Animals deprived of vitamin B show a number of symptoms of disordered function. One of the earliest and one of most outstanding is the loss of appetite, a fact it is important constantly to remember because failure to do so has led to misunderstanding in the past. Many of the lesions which have been described as occurring in the "beriberi" animal are in fact attributable to the wastage consequent on the partial starvation. McCarrison has examined very carefully the condition produced in birds by deficiency of vitamin B; the "avian polyneuritis" of the earlier investigators. He distinguishes two types of the disorder which he terms polyneuritis columbarum and beriberi columbarum. The former is characterized by anorexia, asthenia, symptoms referable to disordered functions of the central nervous system, and by atrophy of most of the organs, excepting the adrenals which may show enlargement. The "beriberi"-like condition is marked by enlargement and dilatation of the heart. A high proportion of the cases show edema and ascites. The relation of these two apparently clinically distinct conditions to the recognized forms of human beriberi is outside the scope of our present discussion.

The loss of appetite has been correlated with some certainty with decreased secretions of the gastric and other digestive juices and there is quite an amount of evidence that vitamin B₁ may play a specific rôle in regulating these secretions. Important as inanition may be in producing the general picture presented by the "beriberi" animal, there are symptoms which have been more sharply correlated with the vitamin deficiency. One is the condition of "polyneuritis." Woollard has made a careful investigation of the nervous system in several species in experimentally produced "beriberi." He could find no evidence of severe degeneration of the peripheral nerves which has often been described. Most of these reports he is inclined to attribute to faulty histologic technic. He himself could find only slight signs of loss of structure in the nerve endings and even these seemed to be as common in simple inanition as in uncomplicated B deficiency. The experimental study of which this investigation formed a part led to the conclusion that the cause of the convulsions seen in "polyneuritis" pigeons and rats is a local disorder of the central nervous system.

The fact that they could be produced by other means than B deficiency, most simply by sublethal doses of various poisons and narcotics, suggested that in "beriberi" a toxic substance might also be responsible. Now it must be recalled that Eijkman as far back as 1896 thought that the disorder of metabolism underlying "beriberi" was the formation of a toxic substance related to carbohydrate breakdown. Moreover, we must call to mind the many occasions on which workers have been led to suspect that "vitamin B" plays a part in carbohydrate metabolism. All sorts of suggestions bearing on this are

to be found in the literature. Many say that the oxidative powers of the organism are depressed in B deficiency, particularly those by which carbohydrates are utilized.* Some find that the development of the symptoms of B deficiency is related to the character of the carbohydrate and the degree to which it is utilized. Others find evidence of incomplete oxidation of sugars in the animal deprived of B.

It has been possible to show with some certainty that no significant fall in the oxygen consumption of resting rats fed on diets devoid of vitamin B₁ takes place until the final and short premortal phase of the decline; and that, even in that period, the reduction is attributable to inanition rather than to the vitamin deficiency.⁴¹ Moreover, no loss of oxidative activity in liver and muscle tissues was detected by measuring the oxygen utilization or their ability to reduce methylene blue under anaerobic conditions. These experiments raised doubts whether vitamin B₁ plays any direct part in tissue oxidations. A somewhat conflicting state of affairs has been resolved in a highly satisfactory manner by a series of investigations in the Biochemical Department at Oxford by Professor Peters and his colleagues. Their first important observation was that a definite increase in lactic acid in the brains of pigeons can be associated with symptoms of opisthotonos in the terminal stages of B₁ deficiency and that the rise is not a consequence of the high blood sugar concentration which may then be prevailing. This discovery suggested that the symptoms in the pigeon might be associated with a failure of the oxidative mechanism of the brain—or even of one small region of it—which might well not be detectable by examining the oxygen consumption of the whole animal.⁴²

This hypothesis has been fully supported by extension of the investigations.⁴³ The curious condition of opisthotonos shown by B-deficient birds is now related with certainty to lesions—biochemical lesions—in areas of the brain. The nature of the disorder has been studied and is gradually being revealed. The mechanism for dealing with lactic acid seems to be deranged in the absence of B₁. Addition of B₁ in the form of highly refined concentrates restores the oxygen consumption of the brain of affected birds to levels almost normal, while at the same time the accumulation of lactic acid disappears. The remarkable fact is that this effect can be readily demonstrated with *in vitro* preparations.

It is very important to note that this reduced metabolic activity of the brain is not due to inanition but is specifically related to the shortage of vitamin B₁. In this we have, at long last, a ray of hope to encourage us to attempt the disentangling of the confused problem of the relation of “avian polyneuritis” to human beriberi. The head retraction in pigeons and other birds, which is so familiar as a text-

* There are many statements that oxidation is depressed in B deficiency and others to the effect that it is unchanged. Westenbrink (*Arch. Nedrl. Physiol.*, 17, 239 (1932)) has criticized this work keenly and has shown that of fifteen workers in the former category only two have actually shown a difference between the normal and the deficient animals which can be accepted as significant.

book illustration, is, as has already been remarked, far from being a specific consequence of vitamin B₁ deficiency. As Hess pointed out, it may be produced by sublethal doses of cyanides and it has been observed to occur in birds deprived of food but receiving daily doses of vitamin B.⁴⁴ It is clearly a nervous disturbance of central origin which can be provoked in more than one manner.

Chemical Nature.—It is over twenty years since Funk isolated from rice-polishings and from yeast a crystalline substance which he believed to be a salt of the antiberiberi vitamin. The method of its separation as well as certain of its properties suggested that the compound might be the nitrate of a base, possibly related to the pyrimidine group.⁴⁵ Various formulae were put forward (C₁₇H₁₈O₂N₂, later C₂₆H₂₀O₉N₄) but before long fears were expressed that the product was merely an inactive crystalline compound contaminated by a trace of the vitamin.

Such suspicions were deepened when other investigators isolated materials which although dissimilar in character were also effective in relieving the so-called "polyneuritis" of vitamin B deficiency in animals. In some cases these preparations were more potent than the crystals isolated by Funk.

In 1926 the position was radically altered by the announcement that two scientists, Drs. Jansen and Donath working in the Dutch Government service in Batavia, had isolated from rice-polishings a crystalline preparation showing a physiological activity of a far higher order than any material previously described.⁴⁶ A long and troublesome method of extraction had yielded a crystalline hydrochloride representing by its activity a very small proportion of that originally present in the polishings. It was stated that the crystals melted at 250° C. and that the analyses suggested the formula C₆H₁₀ON₂. A somewhat superficial examination of their properties led the discoverers to advance tentatively the view that the molecule might contain a glyoxaline nucleus.

It was clear to all that a highly active material had been obtained in crystalline form, but many authorities, recalling more than one similar occurrence in the past, were dubious of its purity. The problem has been attacked by other investigators; most of those who have essayed to repeat the method of isolation described by the Dutch investigators were able, wholly or in part, to confirm the essentials. Most of these efforts were, however, useless from the point of view of establishing the purity of the crystalline hydrochloride for they were not planned on a scale large enough to yield material sufficient for adequate purification. The one investigation which produced a reasonable amount of material—1.3 Gm. of crude crystals from more than 50 tons of rice-polishings—provided grounds for believing that the product of Jansen and Donath was not homogenous.

Then, a profound sensation disturbed the field. A crystalline material, highly active and closely resembling Jansen and Donath's sub-

stance, had been isolated by a group of workers in the laboratory of Professor Windaus at Göttingen and had been found to contain sulfur.⁴⁷ Soon the fact was confirmed and, moreover, proof was obtained that the preparations of the Dutch investigators contained this element. A new formula was advanced by this group of investigators and, before long, others had revised the analytical figures for their own preparations. The results available at the moment are unsatisfactory in that there is a lack of uniformity between the various materials.

| <i>Observer.</i> | <i>Formula.</i> |
|----------------------------|-----------------------|
| Windaus <i>et al</i> | $C_{12}H_{17}ON_3S$ |
| van Veen..... | $C_{12}H_{20}O_2N_4S$ |
| Ohdake..... | $C_{12}H_{16}O_2N_4S$ |

These differences are in themselves disquieting, but when we come to consider the evidence regarding homogeneity which has been obtained from the biological side we feel even less inclined to accept the view that the vitamin has been isolated in the pure state. O'Brien and Peters have recently reported a comparative test carried out under identical conditions on some concentrates prepared in the laboratory at Oxford and on crystalline preparations made by the German and Dutch investigators. The results of these leave little doubt that the former preparations possess an "antineuritic" potency definitely higher than that of the material supplied by Tschesche or Jansen.

From both the chemical and physiological evidence we are justified, therefore, in hesitating to accept the crystals of Jansen and Donath and those of later investigators as pure. If additional warning were necessary it would be provided by the information yielded by a physical method of investigation, namely, the use of the spectroscope. Several observers have recorded absorption bands exhibited in the ultraviolet spectrum by vitamin B preparations but, once again, we encounter discrepancies when the statements are compared. Mukherjee found that crystals similar to those of Jansen and Donath showed marked absorption at 3900–3300 Å. Heyroth and Loufbourow drew attention to the important fact that crude preparations of the vitamin may contain purine and pyrimidine compounds, many of which show strong absorption in the region of 2600 Å where Windaus had observed a band for his vitamin preparation.

They believed, nevertheless, that in such preparations a correlation could be traced between the curative action on animals and the intensity of the absorption at this part of the spectrum. Guha also recorded the band with a maximum at 2600 Å for an electrodialyzed fraction from yeast, but drew attention to the fact that a more potent extract which had been prepared from rice-polishings showed only a very slight absorption at that point.

The matter has been more fully studied recently by Peters and Philpot who employed the crystalline preparations which have already been referred to as being more active than any hitherto described.

These materials show a maximum absorption not at 2600 Å—actually there is only a slight hump on the curve at this point—but at 2450–2490 Å. This band was not correlated with physiological activity.

Only one conclusion can be drawn from this evidence. The crystalline preparations, supposedly of pure vitamin B₁ are not homogeneous and the vitamin has yet to be isolated.

As we are dealing with the contributions made by the spectroscopist to the chemical study of vitamin B₁ it is appropriate that reference should be made to the claim recently made by Guha and Chakravorty that they had converted adenine salts into vitamin B₁ by exposing them to ultraviolet light. The claim was obviously of outstanding importance. The conversion of adenine chloride into the vitamin not only revived the old ideas of the relation of the active substance to the purines and pyrimidines, but put beyond question the possibility of sulfur being a part of the molecule. Unfortunately it has not been confirmed.

Nothing more can usefully be said at the moment about the chemical nature of B₁. The stupendous task of preparing sufficient of the highly potent concentrates to enable a satisfactory chemical purification to be achieved must be faced. Until then we can only safely conclude that the most active preparations hitherto obtained are impure. Even the nature of the inactive "carrier" is unknown, although some of the evidence indicates the presence of a ring system resembling that found in pyrimidines.

Vitamin B₁ Content of Foodstuffs.—This vitamin is found in significant amounts in green plant tissues where, presumably, it is synthesized, but these foodstuffs are not, however, usually regarded as important sources of the body's requirements. The richest plant foods are in the cereal grains in which the vitamin is associated mainly with the embryo. It is commonly believed that the deficiencies of white flour are of no practical importance in human nutrition because they are usually made good by the virtues of other components of a mixed diet. This fallacy has been exposed by the interesting studies by Eddy of everyday diets of the city workers in the United States. There are actually grounds for believing that the diets of the populations of our large towns are suboptimal in respect to vitamin B₁ and, moreover, that a proportion, possibly large, of the disorders of the alimentary tract which are so painfully common today is definitely attributable to this deficiency.

The vitamin is reasonably stable to the modern processes of cooking and preserving foods although the older methods of canning often caused considerable loss. Apart from cereal grains and the pulses, which are also of value, a rich source of B₁ can be obtained in the form of yeast and yeast extracts. The vitamin does not appear to be synthesized by the common industrial yeasts—some species do actually make B₁ when grown in an artificial medium composed of sugar and inorganic salts, *e. g.*, *saccharomyces logos*—rather does the evidence

from the experimental work tend to support the view that the B₁ content of the yeast is derived from the medium. A culture of *S. cerevisiae* grown in B₁-rich malt extract yields a yeast of much higher vitamin content than one grown in a relatively deficient molasses medium. The animal foodstuffs are not of particular value as a source of B₁, excepting, perhaps, eggs, the yolks of which may contain a fair amount.

Cow's milk is of great interest when its content of B₁ is considered because, although the amount present is small by comparison with other foods, it is curiously unaffected by changes in the diet of the animal. An investigation of this unexpected finding led to the discovery that the content of the rumen contributes appreciably to the vitamin B₁ supplies of the cow, primarily through the synthetic activities of certain micro-organisms which flourish there. One of these, a flavobacterium, was found to produce considerable amounts of B₁ when grown in suitable culture media.

VITAMIN B₂ (VITAMIN G)

Physiological Function.—This vitamin has been given a variety of names, most of which relate it to pellagra or to the skin lesions (dermatitis) which may be exhibited by the animal deprived of it. The experimental studies of Goldberger and his colleagues form a remarkable piece of pioneer work on the cause of the curious disease known as pellagra. The earliest of this series of investigations revealed the disease as being clearly a consequence of the consumption of an ill-balanced diet.⁴⁸ At first these workers were inclined to suspect that the chief defect of diets which produced pellagra was insufficient protein of high biological value; an opinion reached independently by Wilson as a result of his studies in Egypt. A few years later Goldberger's researches led him to discover interesting resemblances between the picture presented by pellagra in man; that shown by rats deprived of vitamin B₂ and the condition in dogs, also produced by faulty diet, known as "black tongue."

"Since the experimental pellagra-like condition in the albino rat appears to be due to a deficiency of the same food essential as in experimental black tongue, that is to say, since these two conditions appear to be etiologically indistinguishable, proof of the identity of black tongue and pellagra is also, of course, proof of the fundamental identity of the condition in the rat and the disease (pellagra) in man."⁴⁹

Many accepted this view and Goldberger's designation of the vitamin as the pellagra preventive was widely accepted. Today the pendulum is tending to swing back and much doubt is expressed whether human pellagra can properly be described as a vitamin deficiency disorder. The parallel between the distribution of this factor (B₂) which protects the rat against the onset of a dermatitis and that of the "antiblack tongue" substance protective for dogs is very close, but there are a few discrepancies when we attempt to extend the com-

parison to cover the distribution of the antipellagra (human) substance.

Lack of agreement is even more apparent when we consider the character of the lesions shown by the B_2 -deficient rats and of those which are symptomatic of human pellagra. Probably, as is so often the case in similar disputes, the truth lies somewhere between the two extremes. Indeed, Wilson's recent analysis of the evidence indicates very strongly that the chief dietary fault associated with the appearance of pellagra is, as Goldberger at first suspected, deficient protein of high biological value. An inadequate supply of the factor B_2 may be a contributory factor and, possibly, an essential one. Discussions of the relation of B_2 to human pellagra often disregard the important fact that the disease is sharply localized in the areas in which maize is the staple food (northern Italy, Rumania, south Russia and the "corn belt" of the U. S. A.). Aykroyd has found it necessary to draw attention once again to this fact. He has shown that although whole wheat is richer in B_2 than whole maize, yet the endosperms of the two grains have much the same low value.

It is also amusing to note that an old theory regarding the etiology of pellagra, which was somewhat abruptly rejected by the supporters of the "avitaminosis" hypothesis, has recently been revived and is engaging serious attention once again. This is the theory that the spoilage of maize by micro-organisms produces a toxic substance responsible for the appearance of the disease.

Animals deprived of B_2 exhibit disorders other than the loss of hair and dermatitis to which reference has been made. Many cases show a marked degree of anemia and there has been some discussion whether or not the curative properties possessed by liver are attributable wholly or in part to the vitamin. The position is not entirely clear at the moment but the balance of evidence favors the view that the antianemic factor is not related to B_2 .

Another interesting observation, and one which may possibly be of considerable practical importance in human treatment, is that a large proportion of rats deprived of B_2 sooner or later exhibit cataracts.

Chemical Nature.—Several investigations of the chemical nature of vitamin B_2 are on record. Those of Narayanan and of Guha served to show that the active substance is readily removed from solutions by appropriate adsorbents but that it is not an easy matter to elute the adsorbed material. It was suspected from their work that the vitamin might be a complex molecule, and that it was probably neither markedly acidic nor basic.

A position in which further advances appeared to be a matter of considerable difficulty was, however, clarified with extraordinary rapidity by investigations made in the laboratory of Professor Kuhn at Heidelberg.⁵⁰ At first his views introduced a complication because vitamin B_2 was believed to contain two factors separable by adsorption methods. One was termed *vitamin H* (Haut-faktor) concerned

primarily with protecting the animal against the onset of the skin disorder; the second was believed to be required for growth. One gains the impression from late papers that Kuhn is now less certain that two components of B_2 exist.

The growth-promoting fractions of these preparations have been further examined. By adsorption on fullers' earth from acid solution and elution with dilute pyridine or ammonia, these workers have succeeded in preparing solutions of this vitamin which are characterized by a strong yellow-green fluorescence. These solutions showed growth-promoting power which varied directly with the intensity of the coloration and, moreover, loss of color either by action of light—the pigment being light-sensitive—or by chemical treatment was nearly always accompanied by loss of biological powers.

Apparently similar pigment preparations were separated in concentrated form from egg white, muscle tissue, milk and liver. The preparation from egg white promoted growth in young rats when administered in daily doses of 100 γ .

The name of *flavins* has been adopted for this group of pigments; and there seems little doubt that the members are of considerable biological importance. They may be found naturally associated with substances of high molecular weight and one of the association complexes of this type appears to be the interesting oxidation-pigment-enzyme system discovered by Warburg. The flavins themselves appear to be capable of serving as biological hydrogen acceptors. Lactoflavin, the pigment from milk, has been crystallized and in this purified form shows strong B_2 activity. Analyses indicate formulae such as $C_{17}H_{20}O_6N_4$,⁵¹ $C_{16}H_{20}O_6N_4$ or $C_{17}H_{20}O_7N_4$. The corresponding compound from egg white, ovoflavin, is very similar although the identity of the two substances is not yet fully established.

The pigments of this class are converted into other substances by the action of light, and the derivatives, sometimes themselves pigmented, are devoid of growth-promoting powers. An alkaline solution of crude lactoflavin exposed to light yields a pigment $C_{14}H_{14}N_4O_2$ similar to that obtained by Warburg and Christian from the oxidation enzyme of yeast.⁵²

VITAMIN B_4

Reader discovered this vitamin in 1929 by satisfactorily differentiating the symptoms caused by its lack from those of a somewhat similar nature which are attributable to deprivation of B_1 .³⁸ It can be separated from the other components of the "B-complex" by making use of the fact that it is almost completely precipitated by mercuric sulphate under the appropriate conditions. Chemical investigation of this fraction of "B concentrates" resulted in the isolation of a crystalline hydrochloride of a basic substance showing marked B_4 activity.⁵³ The analyses of the purified salt gave the formula $C_4H_4N_4 \cdot HCl \cdot \frac{1}{2}H_2O$, but Tschesche drew attention to the obvious

resemblance which the description of the compound bore to that of adenine hydrochloride.⁵⁴ This identity is now recognized, but the fact remains that the crystals isolated in Oxford possessed biological activity whereas adenine did not.

VITAMIN C

Physiological Function.—For centuries, scurvy was recognized in a vague manner as a deficiency disease. All who desire to get a clear picture of the disorder and of the ravages it may cause should read, not a modern textbook, but the remarkable account published by Lind nearly two hundred years ago. Almost all the fundamental facts on which our present knowledge is founded are set forth there with a lucidity of exposition and a vividness of description that one seldom finds equalled.⁵⁵

The experimental study of the disease was advanced notably when Axel Holst and Fröhlich discovered that young guinea-pigs develop a condition comparable in almost every respect with human scurvy as a result of being maintained for several weeks on a diet of oats and bran.⁵⁶ Hemorrhages occur in many sites, the bones become brittle and fractures are easily come by, the teeth loosen and the costochondral junctions of the ribs are enlarged. Histologically characteristic changes in the rib junctions and the teeth can be demonstrated. In the former site there is disordering of the rows of cartilage cells and of the trabeculae. In the marrow cavity there is increased blood, and there may be hemorrhages in the vicinity of any injuries to the weakened bone.

The changes in the teeth are of particular interest in view of their value as a means of estimating the degree of deprivation which the animals have suffered: They are of the nature of a fibroid degeneration of the pulp. The hemorrhages which are so symptomatic of scurvy have been attributed to a frailty of the capillary wall brought about by the deficiency of vitamin C. Hess and Fish introduced in 1914 a "capillary resistance" test designed to detect latent scurvy. This test measures the ability of the surface capillaries to withstand increased intravascular pressure brought about by a tourniquet. Employing a test of this type, Göthlin reports having found that children inhabiting the northern parts of Sweden tend to show a decreased capillary resistance toward the end of the long winter and that recovery usually takes place in the summer.

The part played by vitamin C in assisting to maintain a normal state of affairs in the body is unknown. It has long been known that some species are unaffected by being fed for long periods on a scorbutic diet, whereas others soon develop scurvy. The rat and the dog belong to the former category; man and the guinea-pig represent the latter. It has recently been discovered that in all species yet examined there are normally considerable amounts of vitamin C in the adrenal glands. When animals are fed on a diet deficient in this

factor either the stores in the adrenal become depleted or they remain at a normal level. It can be assumed, therefore, that the species which are prone to develop scurvy are those which have lost the power to synthesize the protective substance, whereas, on the other hand, a species such as the rat is independent of exogenous supplies of the vitamin. Since the chemical nature of the vitamin has been revealed by recent researches, there is a tendency to suggest that its powerful reducing properties fit it to play a part in the complex oxidation-reduction processes which form so important a part of the activities of the living cells. There is, however, little of a definite character to accept as yet in this direction.

Chemical Nature.—The story of the chemical investigation of vitamin C is now almost complete. The credit for the pioneer work on the concentration of vitamin C must be given to a large extent to Zilva who for more than ten years gave it his close attention. Using lemon juice as a raw material, he removed the bulk of the organic acids by precipitation of their calcium salts. Additional inactive material was removed by treatment with lead acetate, following which the antiscorbutic factor could be precipitated by the basic acetate. Zilva admitted that the most potent material regenerated from this fraction was probably a complex mixture of substances; but in examining its properties, he discovered that powerful reducing action was frequently, indeed always, associated with physiological activity.

Zilva's studies of the well-known inactivation of the vitamin by oxidation, led him to adopt the view that the reducing substance is important as conditioning the survival of the active factor by protecting it against oxygen.⁵⁷ This interpretation was not wholly acceptable to Tillmans and Hirsch who, from a series of studies on fruit juices, preferred to correlate more directly the antiscorbutic activity of preparations with their reducing powers.⁵⁸ The same tendency was also shown by King and his colleagues, who examining highly active concentrates prepared from lemon juice, were led to suggest that the reducing action is a property of the vitamin itself, which, incidentally, they thought would prove to be an acid.⁵⁹

Let us now digress from this discussion of the direct attack on the chemical nature of vitamin C, in order to refer to an isolated observation which was to prove to be of the greatest possible significance.

In 1920 Dr. Szent-Györgi made observations which led him to suspect that the oxidizing mechanisms of the adrenal cortex might be of peculiar interest. Eight years later the simple experiment which shows that the freshly cut surface of the adrenal cortex rapidly darkens when treated with silver nitrate led him to attempt the isolation of the reducing substance. The effort was successful and a crystalline compound was separated which appeared to be that responsible for the reaction. His examination of its properties suggested that it was a hexuronic acid which readily formed a lactone of the formula $C_6H_8O_6$.⁶⁰

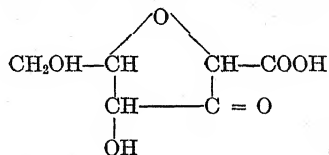
In view of the possible relation of such a compound to the peroxidase system in plants, an examination of cabbage and orange juice was made. From both materials the acid was isolated. It was obvious that such a compound would satisfy the requirements for the reducing principle detected by Zilva's work, and Szent-Györgi remarked, "Indophenol blue is readily reduced by the hexuronic acid, so that it is probable that it was the substance which had been studied by Zilva."

It seems to have been the firm statement of Zilva's opinion that the reducing principle was only the "stabilizer" of the vitamin, which was responsible for delaying the carrying out of direct tests to ascertain whether the "hexuronic acid" crystals possessed antiscorbutic properties. At any rate, such tests do not seem to have been made until two years ago when the dramatic announcement of a positive result was made by Svirbely and Szent-Györgi.⁶¹

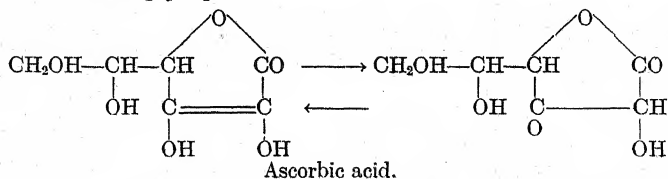
The first examination of the crystals isolated by Szent-Györgi was made by Haworth, Hirst and Reynolds, who reported that it was probably the 6-carboxylic acid of a ketohexose, which did not appear to be *d*-fructose or the ketose corresponding to *d*-galactose. In recognition of the fact that the analysis does not represent the formula of a hexuronic acid but that of a dehydrated molecule, $C_6H_8O_6$, the substance has been renamed *ascorbic acid*.

The study of the oxidation of the acid led Hirst, Cox and Reynolds⁶³ to suggest that it possessed the structure of a diketo-acid showing tautomeric change.

Micheel and Kraft prepared a dimethyl derivative by the action of diazomethane in which one methoxyl group was ester in nature and which was stable to iodine in acid solution. The stability of the *p*-nitrobenzoate of this dimethyl compound to other oxidizing reagents indicated that it is not enolic in character. Evidence was also obtained that one of the oxygen atoms may be involved in a ring structure and accordingly the following formula was advanced:



This formula was considered by Haworth to be incompatible with certain of the chemical evidence and with the crystallographic data.⁶⁴ The work of the Birmingham laboratory led to the following alternative structure being proposed:



This formula accounts satisfactorily for all the known chemical reactions of ascorbic acid. The final chapters of a brilliant record of chemical work on this interesting substance are the papers, one by a large team of investigators in the laboratory of Professor Haworth at Birmingham, the other by Reichstein, Grüssner and Oppenauer which describe the synthesis of *d*- and *l*-ascorbic acid from xylosone.^{65*}

Vitamin C in Foods.—It is almost universally recognized that fresh fruit and vegetables are the chief sources of vitamin C in the diet. The rôle of the vitamin in the plant is unknown but it first appears in seeds during the early stages of the complex process which constitutes germination. The variations in the amount present in the plant tissues during growth, fruition and ripening have not yet been adequately studied but it will now be possible to obtain such information by using the chemical methods of estimation which will doubtless supplant the more tedious animal tests.⁶⁶

At one time it was believed that the antiscorbutic properties of foods were lost with great rapidity during cooking, canning or other forms of treatment. This belief arose from experiments made with products which were made by processes now for the most part obsolete. Hess was one of the first to point out that properly canned tomatoes may be almost as valuable an antiscorbutic factor as the fresh fruit. The careful series of investigations made by Kohman and Eddy has confirmed and extended this opinion and it is now established that many canned fruits and vegetables retain a large proportion of the original vitamin. Fruit juices have been concentrated to a small bulk and even dried with little destruction of C, and such preparations have been employed with considerable success as protective substances in the ration of explorers and others cut off for long periods from supplies of fresh foods.

As regards foodstuffs of animal origin we have, in the first place, milk. The extensive studies of the C content of cow's milk make it reasonably certain that the vitamin is transferred from the diet to the mammary secretion. The antiscorbutic value of milk tends to fall to a minimum at the end of the winter period of indoor feeding on concentrates and hay. In the summer, however, the value rises in proportion to the amount of pasture or fresh vegetable food that is available. Drought or any loss of "freshness" of the pasture causes a fall in the amount of C in the milk. At its best, cow's milk is but a poor supply of vitamin C for infants and it is almost always necessary to supplement it by a recognized antiscorbutic such as orange juice. Modern methods of drying milk cause some but not complete loss of C and much the same can be said of the effect of pasteurizing. One of the interesting developments of the recent researches on ascorbic acid has been the discovery of quite considerable stores in the adrenal glands and the liver. The amounts present in other tissues are probably of a smaller order but quite sufficient to explain the long-known value of freshly killed meat in protecting

* Refer to page 66.

against the onset of scurvy. The history of Arctic and Antarctic exploration provides many examples of protection provided by fresh seal meat.

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BIBLIOGRAPHY

1. T. Percival's Essays, Vol. ii, 4th Edition (London, 1789), p. 359.
2. McCollum, E. V., et al., *J. Biol. Chem.*, **53**, 29 (1922).
3. Fridericia, L. S.: *Am. J. Physiol.*, **73**, 63 (1925).
4. Tansley, K.: *J. Physiol.*, **71**, 442 (1931).
5. Aykroyd, W. R.: *J. Hyg.*, **30**, 357 (1930-31).
6. Mori, M.: *Jahrb. Kinderhkl.*, **59**, 175 (1904).
7. Wright, R. E.: *Lancet*, **220**, 800 (1931).
8. Bloch, C. E.: *Ugeskr. Laeg.*, **79**, 282, 309, 349 (1917); *J. Hyg.*, **19**, 283 (1921).
9. Goldblatt, H., and Benischek, M.: *J. Exp. Med.*, **46**, 699 (1927).
10. Frazier, C. N., and Hu, C. K.: *Arch. Internal Med.*, **48**, 507 (1931).
11. Green, H. N., and Mellanby, E.: *Brit. Med. J.*, **ii**, 691 (1928).
12. Drummond, J. C., Channon, H. J., Coward, K. H., and Baker: *Biochem. J.*, **19**, 1047 (1925); **23**, 274 (1929).
13. Rosenheim, O., and Drummond, J. C.: *Biochem. J.*, **19**, 753 (1925); Carr, F. H., and Price, E. A.: *Biochem. J.*, **20**, 497 (1926).
14. Rosenheim, O., et al., *Biochem. J.*, **26**, 1178 (1932).
15. Karrer, P.: *Helv. Chim. Acta*, **14**, 1036 (1931).
16. Steenbock, H.: *Science*, **50**, 352 (1919).
17. Euler, H. von: *Biochem. Z.*, **203**, 370 (1928).
18. Moore, T.: *Biochem. J.*, **23**, 803 (1929).
19. Kuhn, R., and Brockman, H.: *Ber.*, **66**, 407 (1933).
20. Kuhn, R., et al., *Z. physiol. Chem.*, **221**, 129 (1933); Brockman, H., and Tecklenburg, M. L., *Ibid.*, **221**, 117 (1933).
21. Kuhn, R., and Brockman, H.: *Ber.*, **64**, 1319 (1933).
22. Gillam, A. E., et al., *Biochem. J.*, **26**, 1118 (1932); Kuhn and Lederer, *Ber.*, **64**, 2859 (1932).
23. Macwalter, R. J., and Drummond, J. C.: *Biochem. J.*, **27**, 1415 (1933).
24. Palm, T. A.: *Practitioner*, **45**, 271, 321 (1890); Huldchinsky, K.: *Deut. med. Wochschr.*, **45**, 712 (1919); Hess, A. F., and Unger, L. J.: *Am. J. Diseases Children*, **22**, 186 (1921).
25. Steenbock, H., and Black, A.: *J. Biol. Chem.*, **61**, 405 (1924).
26. Angus, T. C., et al., *Proc. Roy. Soc. (London)*, **108B**, 340 (1931).
27. Windaus, A.: *Proc. Roy. Soc. (London)*, **108B**, 568 (1931); *Ann.*, **489**, 252 (1931).
28. Angus, T. C., et al., *Proc. Roy. Soc. (London)*, **109B**, 488 (1932).
29. Windaus, A., et al., *Ann.*, **492**, 226 (1932).
30. Evans, H. M., and Bishop, K. S.: *J. Metabolic Research*, **1**, 319, 355 (1922).
31. Sure, B.: *J. Biol. Chem.*, **58**, 623, 681 (1923-24).
32. Evans, H. M., et al., *Memoirs of Univ. of California*, **8**, 1927.
33. Beard, H. H.: *Am. J. Physiol.*, **75**, 668 (1925-26).
34. Vogt-Müller, P.: *Lancet*, July 25 (1931).
35. Evans, H. M., and Burr, G. O.: *J. Am. Med. Assoc.*, **99**, 469 (1932).
36. Smith, M. I., and Hendrick, E. G.: *U. S. Pub. Health Reports*, **41**, 201 (1926).
37. Williams, R. R., and Waterman, R. E.: *J. Biol. Chem.*, **78**, 311 (1928).
38. Reader, V.: *Biochem. J.*, **23**, 689 (1929).
39. Chick, H., and Copping, A. M.: *Biochem. J.*, **24**, 1744 (1930).
40. Carter, C. W., Kinnersley, H. W., and Peters, R. A.: *Biochem. J.*, **24**, 832, 1844 (1930).
41. Marrian, G. F., and Drummond, J. C.: *Biochem. J.*, **20**, 1229 (1926); **21**, 1336 (1927).
42. Kinnersley, H. W., and Peters, R. A.: *Biochem. J.*, **22**, 1126 (1929).
43. Kinnersley, H. W., and Peters, R. A.: *Biochem. J.*, **24**, 711 (1930); Gavrilescu, N., and Peters, R. A.: *Proc. Roy. Soc. (London)*, **110B**, 431 (1931); **111**, 391 (1932).
44. Marrian, G. F., et al., *Biochem. J.*, **21**, 1336 (1927).
45. Funk, C.: *J. Physiol.*, **13**, 395 (1911); Drummond, J. C., and Funk, C., *Biochem. J.*, **8**, 598 (1914).
46. Jansen, B. C. P., and Donath, W. F.: *Proc. Kon. Akad. Wetens. Amsterdam*, **29**, 1390 (1926).

47. Windaus, A., *et al.*, *Nachr. Ges. Wiss. Göttingen*, **111**, 207 (1931).
48. Goldberger, J., *et al.*, *U. S. Pub. Health Reports*, **35**, 648 (1920).
49. Goldberger, J., *et al.*, *U. S. Pub. Health Reports*, **40**, 1087 (1925).
50. Kuhn, R.: *Ber.*, **66**, 317 (1933).
51. Kuhn, R., *et al.*, *Ber.*, **66**, 1034 (1933).
52. Kuhn, R., *et al.*, *Biochem. Z.*, **258**, 496 (1933).
53. Barnes, H., *et al.*, *Biochem. J.*, **26**, 2035 (1932).
54. Tscheche, R.: *Ber.*, **66**, 581 (1933).
55. Lind, J. A.: *A Treatise on the Scurvy* (1757).
56. Holst, A., and Fröhlich, T.: *J. Hyg.*, **7**, 634 (1907).
57. Zilva, S. S., *et al.*, *Biochem. J.*, **24**, 1687 (1930).
58. Tillmans, J., and Hirsch, P.: *Z. Untersuch. Lebensm.*, **63**, 1, 21 (1932).
59. King, C. G., *et al.*, *J. Biol. Chem.*, **87**, 615 (1930); **94**, 483 (1931).
60. Szent-Györgi, A. von: *Biochem. J.*, **22**, 1385 (1928).
61. Svirbely, J. L., and Szent-Györgi, A. von: *Nature*, **129**, 690 (1932); *Biochem. J.*, **26**, 865 (1932).
62. Waugh, W. A., and King, C. G.: *J. Biol. Chem.*, **97**, 325 (1932).
63. Hirst, E. L., Cox, E. G., and Reynolds, R. J. W.: *Nature*, **129**, 576 (1932).
64. Howarth, W. N., *et al.*, *J. Soc. Chem. Ind.*, **52**, 221 (1933).
65. Howarth, W. N., *et al.*, *J. Chem. Soc.*, 1419 (1933); Reichstein, T., Grüssner, A., and Oppenauer, R.: *Helv. Chim. Acta*, **16**, 1019 (1933).
66. Tillmanns, J.: *Z. Untersuch. Lebensm.*, **60**, 34 (1930); *Biochem. Z.*, **250**, 312 (1932); Harris, *et al.*, *Biochem. J.*, **27**, 590 (1933).

RECENT REVIEWS ON VITAMINS AND RELATED SUBJECTS

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Goldlatt, H.: *Ergeb. Allg. Path. u. Path. Anat. des Menschen u. der Tiere*, p. 58, 1931.

(This is a very complete review giving no less than 2723 references to the original literature.)

Pathologic Calcification

Barr, D. P.: *Physiol. Rev.*, **12**, 593 (1932).

Parathyroid Glands

Collip, J. B.: *Physiol. Rev.*, **12**, 309 (1932).

Pellagra

Stepp and Voit: *Neue Deutsche Klinik. Ergänzungsband*, **1**, 97 (1933).

Scurvy

Stepp and Voit: *Neue Deutsche Klinik. Ergänzungsband*, **1**, 10 (1932).

Vitamin A and Carotene

H. v. Euler: *Ergebnisse Physiol.*, **34**, 360 (1932).

Vitamins and Hormones

Winterstein, A., and Schön, K.: *Ergeb. Hygiene u. Bakteriologie*, **14**, 436 (1933).

Monographs on Vitamins

Browning, E.: *The Vitamins*. Monographs of Pickett-Thomson Research Laboratory, Vol. I, 1931.

(This volume is of particular value in giving references to much important clinical literature.)

Sherman, H. C., and Smith, S. L.: *The Vitamins*. Am. Chem. Soc. Monograph, 2nd Edition, 1931.

The Vitamins, A Survey of Present Knowledge. Medical Research Council, Special Report No. 167. H. M. Stationery Office, London, 1932.

Aykroyd, W. R.: *The Vitamins and Other Dietary Essentials*, 1933.

McCarrison, R.: *Studies in Deficiency Diseases*, 1921.

Hess, A. F.: *Scurvy, Past and Present*, 1920.

Hess, A. F.: *Rickets, Osteomalacia and Tetany*, 1930.

CHAPTER X

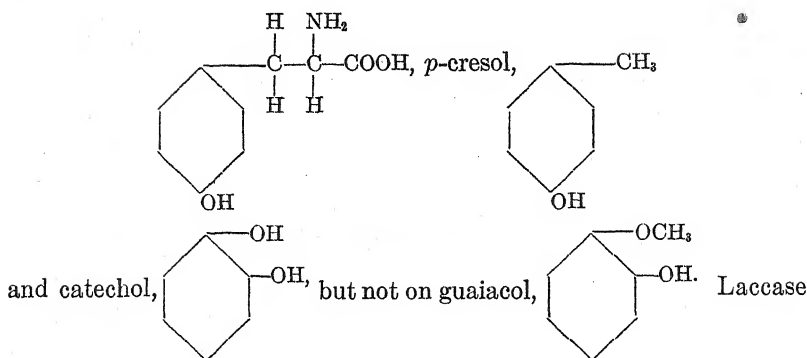
ENZYMES

ENZYMES may be defined as catalysts which are elaborated by living cells and which may act independently of the life processes of the cells. For the present we are forced to classify and study them by their activities and by the effects of various influences on these activities. It is well to bear in mind that some authorities consider that catalysts may initiate a reaction as well as alter its rate. From this point of view, Bayliss¹ has defined a catalyst as "a substance which changes the rate of a reaction which is actually in progress, or which is capable of proceeding without any supply of energy from without, if certain resisting influences are removed." This conception of catalysts as initiators as well as accelerators (or retarders), may quite properly be applied to enzymes.

The importance of enzymes in physiology is unquestioned. Willstätter,² a leader in this field of investigation, says, "We may regard life as a system of cooperating enzymatic reactions." The hydrolytic processes in digestion, the oxidizing and reducing reactions in respiration, the disintegrations in cell metabolism—and probably the synthetic processes as well—all depend upon these catalysts for their acceleration. The rate of these reactions is modified by various factors, including hydrogen ion concentration and the ratio of substrate to products; the regulation of the rate *in vivo* may be under the control of nervous, hormonal, or other physiologic factors.

Specificity.—The enzymes are specific to a high degree. That is, a given enzyme will attack only a certain compound or group of compounds. Since this "attack" is undoubtedly always directed at a definite type of linkage in the molecule, there are many apparent exceptions to this rule of specificity. However, it may be stated definitely that, on the one hand, broad boundaries are never crossed; lipases do not split proteins, carbohydrases do not saponify fats, etc. On the other hand, there are many examples of very specific activity. Urease is one of the comparatively few enzymes which act upon only one substance. From yeast Grassman and Dyckerhoff separated a dipeptidase and a polypeptidase. The former attacks dipeptides but none of their derivatives; apparently free NH_2 and COOH groups must be present in order that its activity may be manifested. The polypeptidase will not hydrolyze dipeptides, but does split tripeptides and polypeptides.

Some of the oxidizing enzymes are very interesting from this standpoint. Tyrosinase acts on tyrosine,



affects catechol and guaiacol, but not tyrosine or *p*-cresol. There are at least two types of maltase, or α -glucosidase. One, found in yeast, is less specific and hydrolyzes both maltose and various α -glucosides. Another, in the fruit of *Solanum indicum*, splits maltose easily, but only the more readily hydrolyzable α -glucosides. The former has been termed by Kleiner and Tauber³ a "true α -glucosidase"; the latter a "pseudo α -glucosidase."

According to Dakin, liver lipase saponifies the *d*-mandelic acid esters much more rapidly than it does the levo-, and stereochemical selectivity is also shown in the action of erepsin, which hydrolyzes glycyl-*l*-leucine while hardly attacking glycyl-*d*-leucine. The phosphatases, obtained from mammalian sources, hydrolyze any phosphoric ester in which only one of the hydroxyl groups is esterified by an alcohol; but attack very slowly, or not at all, those in which two hydroxyls are involved. Furthermore, the phosphatase of red cells acts more energetically on α - than on β -glycerophosphate.⁴

Haldane⁵ has quite legitimately extended the term "specificity" to include ways in which different enzymes attack the same substrate. For example, the zymase of yeast attacks glucose with the production of alcohol and carbon dioxide; muscle zymase breaks the same molecule down to lactic acid. One type of saccharase selects the glucose half of the sucrose molecule for the point of attack, while another selects the fructose portion of the same molecule.

Properties.—Enzymes either are colloids or are so closely associated with colloids that they have not been isolated in a noncolloidal state. Therefore their physical and chemical properties are those of colloids. In general, they dialyze very slowly or not at all; they are changed, injured, or denatured, by heat; and they may be precipitated by various colloidal reagents.

The property of *not dialyzing* readily is employed in the purification of certain enzymes in order to free them of inorganic salts, organic compounds of small molecular size, or to change the hydrogen ion concentration. In some cases, as, for example, liver lipase, this has resulted in inactivating the enzyme by removing an essential in-

organic fraction, a "coenzyme." In the case of rennin,⁶ dialysis must be employed sparingly because this enzyme has a comparatively small molecule and goes through a parchment membrane. Trypsin, obtained by autolysis, dialyzes even more rapidly.

Being colloids, enzymes may, of course, act as "protective colloids," and it has even been shown⁷ that the enzyme trypsin may act as a protective colloid for urease (which is unstable in the absence of a suitable colloid).

Most enzymes are *inactivated by heating* above 70° C. An interesting exception is trypsin,⁸ which may be heated nearly to boiling for a short time, with very little loss in activity, if the solution is acid in reaction. Trypsin, however, gradually loses its activity when dry. Other enzymes are unstable in solution. Still others (such as rennin) lose some of their activity while being dried; but in that state remain fairly constant. This same enzyme, rennin, is sensitive to shaking; and there seems to be a general opinion that violent agitation destroys enzymes.

Visible light rays do not have much effect on most enzymes; but ultraviolet light has been shown to have an inhibitory action in certain instances.⁹

The *optimum temperature* has been determined for many enzymes. For most animal enzymes it is around 40° C., but for plant enzymes it is usually higher—a plant rennin, with an optimum temperature of 80°–85° C., has been reported by A. Bodansky.

The commonest *solvents* employed in the preparation of enzyme solutions are water, dilute ethyl alcohol, glycerol and dilute acids and alkalis. From these solutions, the enzymes may be precipitated by strong alcohol, acetone and by various concentrations of ammonium sulfate and other salts. They may also be readily adsorbed. These properties are made use of in concentrating and purifying enzymes.

An interesting (and physiologically important) property of enzymes is the *reversibility* of their reactions. This has long been known for the lipases and esterases and has been assumed to be applicable, theoretically at least, for all enzyme reactions. It cannot be demonstrated in many cases; as, for example, in those reactions in which intramolecular rearrangements take place concomitantly with the primary reaction. Falk¹⁰ lists the following enzymes as having been definitely found to accelerate synthetic changes: Lipase, emulsin, trypsin, pepsin, kephirlactase, maltase, and oxynitrilase.

The optimal conditions for the synthetic action of an enzyme are sometimes quite different from its optimal conditions for cleavage. For example, Wasteneys and Borsook¹¹ found the optimal pH for the synthesis of protein to be 4, whereas the most rapid peptic digestion takes place at about pH 1.4. This synthesis of *plastein* from a peptic digest of egg albumin proceeds with extreme rapidity. Kay states that the optimum pH is about the same for synthesis and hydrolysis by phosphatase; *i. e.*, about 9–9.4.

The *velocity of enzyme action* depends upon the concentration of substrate, the concentration of enzyme, and the temperature. Other factors, which vary more according to the specific enzymes concerned, are the hydrogen ion concentration and the presence or absence of activating or inhibiting substances, including the products of the reaction. At very low concentrations of the substrate, the velocity increases with increasing concentration, then usually remains constant, and eventually decreases as the substrate becomes more concentrated. Although the velocity is not directly proportional to the concentration of enzyme, it is very definitely affected by it.

The velocity of enzyme activity is markedly affected by the hydrogen ion concentration. Some enzymes are inhibited; some are reversibly, and others irreversibly inactivated by unfavorable reactions. The optimum pH for various enzymes is given in the table on page 311. In many cases these figures are approximate only, and further work is needed before accurate data can be assembled. The optimum reaction for a given enzyme may vary, for example, with the presence of small amounts of different salts, or with the buffer employed.

Preparation.—The modern methods of obtaining concentrated enzyme preparations fall into two general categories. The first is the method initiated by earlier workers and developed by Willstätter and his associates. This consists in causing the adsorption of the enzyme by some suitable colloid, and the subsequent freeing or elution of the enzyme. The second method, or rather group of methods, is that used chiefly by various American investigators. The enzymes in these procedures are dissolved in suitable solvents and concentrated by "salting out," or by precipitation with various reagents. Changes in hydrogen ion concentration and temperature, dialysis, and other measures are employed either to rid the medium of impurities or to concentrate or to crystallize the enzyme. Frequently a combination of the two procedures has been used.

If the enzyme to be isolated is present within the cells of the animal or vegetable tissue, it must first be liberated from the cell. This may be accomplished by one or more of the following means: (a) Physical methods, such as chopping, grinding with sand, alternate freezing and thawing, or crushing; (b) preliminary weakening of the cell wall by extraction of lipids; (c) simple extraction of the enzyme with suitable solvents; (d) autolysis; and (e) the action of enzymes (other than autolytic) upon the cell wall. Once the enzyme is liberated, the process of isolation may begin immediately or the material may be dried first. The latter procedure is often used because, in the drying process, a portion of the protein present is denatured and then may be discarded. On the other hand, the drying may have a deleterious effect upon the enzyme, especially if it be carried out in air.

Willstätter's methods have formed the basis of a great deal of current discussion of enzyme problems. The point of view of this

OPTIMUM REACTIONS FOR VARIOUS ENZYMES¹

| Enzyme | Optimum pH | Authority |
|---------------------------------------|------------------|-------------------------------|
| Amylase, salivary..... | 6.1-6.9 | Michaelis and Pechstein. |
| Amylase, pancreatic..... | 7.0 | Sherman, Thomas, and Baldwin. |
| Amylase, malt..... | 4.6-5.2 | Lüters and Wasmund. |
| Catalase..... | 7.0 | Sørensen. |
| Erepsin, intestinal..... | 7.7 | Rona and Arnheim. |
| β -Glucosidase..... | 5.0 | E. Fischer. |
| Lipase, pancreatic..... | 8 | Davidson. |
| Lipase, gastric..... | 4-5 | Davidson. |
| Lipase, salivary..... | 7.6-7.8 | Koldayev and Pikul. |
| Lipase, ricinus..... | 4.7 | Haley and Lyman. |
| Melibiose..... | 5.5 | Tauber and Kleiner. |
| Maltase..... | 6.1-6.8 | Michaelis and Rona. |
| Maltase, <i>Solanum indicum</i> | 5.5 | Tauber and Kleiner. |
| Papain..... | 4-7 | Grassmann. |
| Pectolase..... | 4.5-6.3 | F. Ehrlich. |
| Pepsin (on egg albumin)..... | 1.2-1.6 | Sørensen. |
| Pepsin (on gelatin)..... | 3.0-3.5 | Dernby. |
| Pepsin, synthetic action..... | 4.0 | Wasteneys and Borsook. |
| Phosphatase, bone..... | 9.5 | Robison. |
| Phosphatase, plasma..... | 8.8-9.2 | Kay. |
| Phosphatase, red blood cells..... | 6.0-6.8 | J. Roche. |
| Phosphatase, plant sources..... | 3.4-6.0 | Kay and Lee. |
| Phosphatase, synthetic action.... | 9.4 | Kay. |
| Protease, autolytic (kidney, liver). | 4.5 | Bradley. |
| Rennin..... | 5.4 ² | Fenger. |
| Sucrase, intestinal..... | 6.8 | Euler and Svanberg. |
| Sucrase, yeast..... | 4.4-4.6 | Sørensen; Fales and Nelson. |
| Sucrase, <i>Solanum indicum</i> | 6.0 | Tauber and Kleiner. |
| Trypsin, pancreatic..... | 8.0 | Lundén. |
| Trypsin, synthetic action..... | 5.7 | Wasteneys and Borsook. |
| Tyrosinase..... | 6-8 | Raper. |
| Urease..... | 7.0 | Van Slyke and Zacharias. |

¹ Amplified from Falk¹⁰ and Waldschmidt-Leitz.¹²
² This is really the optimum pH for casein precipitation and is influenced by the iso-electric point of casein.

school is that "there exists but a single method for the isolation of enzymes which is versatile, adaptable, and capable of development; namely, the employment of the adsorption processes based upon small affinity relations or upon residual affinities."¹² The search for adsorbents which are highly selective and for optimum conditions of adsorption has been carried out in a great number of investigations. Various aluminum hydroxides, kaolin, and charcoal have been used. Using different adsorbents—or the same adsorbent at differing hydrogen ion concentrations—enzymes may be separated from each other and from most of the associated substances in relatively high concentrations.

The adsorbent is assumed to hold the enzyme by such a small number of its "affinities" that it can be released by very mild agents. The enzyme is thus freed from its adsorbent by elution. As eluents, dilute electrolytes (such as weak alkalis or phosphates) are employed. These have a slightly greater affinity for the adsorbent than for the adsorbate. It may be redissolved, reabsorbed, and reeluted several times in order to increase the strength of the preparation. Eventually a limit in concentration is reached with a given adsorbent beyond which further use of the particular agent gives preparations which do not increase in strength. This is due to the adsorption of impurities along with the enzyme. Substitution now of an adsorbent of the opposite electrical polarity may favor the concentrating process.

By methods depending chiefly upon solution and precipitation several enzymes have been concentrated—some even in crystalline form. The preparation of crystalline urease by Sumner¹³ is accomplished by the extraction of jack bean meal with dilute acetone at a low temperature. Sharply defined octahedral crystals of a globulin nature and high urease activity are thus obtained. Crystalline pepsin has been isolated by Northrop.¹⁴ It is obtained from commercial pepsin by salting the pepsin out of a sulfuric acid solution by the addition of a saturated MgSO_4 solution. It is then dissolved in alkali, precipitated again with acid, redissolved in warm alkali and allowed to cool very slowly. Crystalline trypsin has been obtained by Northrop and Kunitz¹⁵ by adding saturated ammonium sulfate solution to a concentrated solution of trypsin in one quarter saturated ammonium sulphate. This crystalline enzyme, which is extremely difficult to prepare, is also of a protein nature.

Recently another crystalline enzyme has been prepared from pancreatic tissues by the same workers.¹⁶ It differs from the first product in crystalline form and in proteolytic power. *Chymotrypsinogen*, as it has been named, is converted into *chymotrypsin* by minute amounts of trypsin. Chymotrypsinogen is less active than crystalline trypsin in digesting hemoglobin, casein or gelatin, but more active in clotting milk. A crystalline amylase was prepared by Caldwell, Booher and Sherman in 1931 from buffered alcohol-water solutions of concentrated pancreatic amylase. The exact method has not been described.

Thus far the only crystalline enzyme the preparation of which has been successfully repeated is crystalline urease.

An extremely concentrated rennin solution may be prepared by fractional iso-electric precipitation of a dilute HCl extract of the fourth stomach of the calf.⁶ Although not crystalline, the preparation obtained by this method must be almost pure, since it contains practically no pepsin.

Hammarsten maintained for years that rennin and pepsin are separate enzymes; while Nencki and Sieber, Pekelharing, Pavlov, and others were equally insistent that the milk-curdling power of extracts of gastric mucosa is merely one of the first phases of peptic activity, and that rennin does not exist. The principal mode of approach was to study the quantitative effects of a given inhibitory influence on the peptic and rennet powers of a preparation. If the effects were paralled it was assumed that one enzyme caused both actions. Now, however, a comparison of purified rennin with crystalline pepsin shows that they are quite different chemically. The milk-clotting power of pepsin is, to be sure, a phase of that enzyme's activity; but rennin, a much more powerful milk curdler, with extremely little peptic power, also exists. Apparently, animal rennin is found almost exclusively in the young, and pepsin, free from rennin, in the adult.

Rennin appears to be a thioprotease, pepsin a simple protein. They differ radically in their solubilities, iso-electric points, coagulability, protein color tests, dialyzability, and finally in their elementary composition. Moreover, rennin is rapidly digested by pepsin. In fact, the latter enzyme may be freed from all traces of rennin by this means.

ELEMENTARY COMPOSITION OF HIGHLY PURIFIED ENZYMES

| | C | H | N | S | P | Cl | Ash | Authority |
|--|-------|------|------|------|-------|------|------|----------------------|
| Crystalline urease. . . . | 51.6 | 7.1 | 16.0 | 1.2 | | | 2.0 | Sumner. |
| Crystalline pepsin. . . . | 52.4 | 6.66 | 15.4 | 0.85 | 0.078 | 0.21 | 0.47 | Northrop. |
| Crystalline trypsin. . . . | 50.0 | 7.2 | 14.9 | 1.10 | 0.00 | 2.88 | 1.2 | Northrop and Kunitz. |
| Non-protein non-crystalline trypsin. | 49.45 | 6.24 | 11.2 | 0.25 | 0.00 | 0.28 | 6.49 | Kleiner and Tauber. |
| Purified rennin. | 61.33 | 7.02 | 14.4 | 1.19 | 0.00 | 0.00 | 0.40 | Tauber and Kleiner. |

The drying of purified enzymes presents an additional problem. Desiccation of rennin, for example, *in vacuo* leads to a loss of half of its potency. Sometimes the use of a stream of warm air is less harmful; and, in the case of urease, lipase, and some other enzymes, dehydration by acetone is least objectionable. If the enzyme solution be placed in a dialyzing bag and hung in the air at or slightly above

room temperature, moisture will be lost at a rapid rate without deleterious effect upon the enzyme.¹⁷

Mechanism of Enzyme Action.—Several theories regarding the mechanism of enzyme action have been proposed. Bayliss¹ suggested the necessity for the formation of adsorption compounds between enzyme and substrate as a prerequisite for enzyme activity. His theory implies that the changes brought about occur at the interface between the phases of a heterogeneous system. Furthermore, he maintains that all the constituents of the chemical system are condensed on the surface of the enzyme phase, and hence equilibrium is established comparatively rapidly.

Another type of enzyme-substrate combination is postulated by Michaelis. This type is assumed to be reversible, but not only is there a reversible combination between the enzyme and the substrate, but also between the enzyme and the products of the reaction. The well-known fact that products of an enzyme reaction tend to inhibit the progress of the reaction harmonizes well with this hypothesis.

In his studies of purified pepsin and trypsin, Northrop has found that the idea of a combination between enzyme and substrate is not necessary. These enzyme reactions proceed in accordance with the law of mass action. However, he explains part of his results by assuming that reversible combinations of the enzyme with the products of the reaction do occur. Others have likewise interpreted their results in harmony with the mass law, but deviations from the law are always found.

Activation.—Several enzymes are known to exist in an inactive form and to require for their activation either the presence of some specific (or nonspecific) "activator" or of a decided change in hydrogen ion concentration. Pepsin is present in the gastric mucosa as pepsinogen. The hydrochloric acid of the gastric juice transforms it into pepsin. This occurs when a pH of 1.6 has been reached.¹⁸ The actual occurrence of pepsinogen can be demonstrated quite easily by subjecting both neutral and acid extracts of the mucosa to the influence of a weak alkali. Upon adjustment of the pH to the optimum for pepsin, only the extract which was originally neutral (*i. e.*, the pepsinogen solution) will show peptic power.

A precursor of rennin can likewise be obtained from the fourth stomach of the calf.⁹ This has very slight milk-curdling powers, which are markedly increased by the action of acid; but in this case a pH of only 3.6 is required. Again, the *zymogen* (the precursor of the enzyme) and enzyme may be differentiated by their behavior toward a decrease in hydrogen ion concentration, rennin being unstable at a pH of over 6, while prorennin is prepared at pH 7.4.

Pancreatic juice, as obtained directly from the pancreatic duct, has no or only slight action upon most proteins, but when mixed with an extract of the intestinal mucosa it becomes strongly proteolytic. The intestine contains *enterokinase*, a specific activator for trypsinogen.

Trypsinogen may also be activated spontaneously upon allowing inactive pancreatic preparations to stand.⁵ From the trypsin, activated in either way, Waldschmidt-Leitz has been able to separate the kinase. Evidence has been brought forward to show that this kinase is not an enzyme, as was formerly believed, but forms an addition product with the inactive, or at best, feebly active enzyme. Apparently the enterokinase, in an inactive stage, is also secreted by the pancreas and accompanies this inactive trypsinogen into the duodenum.* The pro-kinase is converted into kinase by the cells of the intestinal mucosa. Obviously this elaborate mechanism prevents the formation of active trypsin within the pancreas and thus protects the organ against self-digestion.

As stated previously, the dialysis of certain enzymes inactivates them. They may then be reactivated by addition of some of the dialyzed electrolyte, which is called a *coenzyme*. The only essential difference between a kinase and a coenzyme is that the former is colloidal and the latter crystalloidal. The kinases, as might be expected, are more specific than the coenzymes. Some of the activators which have been demonstrated are chloride and bromide ions for pancreatic amylase, bile salts for lipase, and glutathione for glyoxylase¹⁹ and other enzymes (such as papain and kathepsin). Activators may, in some cases, increase the rate of activity of already active enzymes. For example, the addition of hydrogen cyanide increases tremendously the activity of papain at the optimum of pH, and phosphatase from red blood cells may have its effect increased 3000 per cent by the addition of its coenzyme, magnesium ion.

The studies on zymase in the laboratories of Meyerhof, of Harden, and of Euler have brought out some interesting points in the field of activation, particularly with regard to the rôle of phosphates.²⁰ The fermentation of sugar by living yeast proceeds ordinarily at a rapid pace, and this is not materially changed by the addition of phosphate. The press-juice, however, ferments quite slowly; but by the addition of phosphate the rate may be increased to approximately one half the rate of living yeast.

Inorganic phosphates are required for the formation of phosphoric acid esters of the fermentable sugar. Here the substance which increases the rate of reaction cannot be considered an activator of the enzyme, but rather something needed to render the substrate available for the reactions. For zymase activity a coenzyme is also needed.²¹ Mixtures of apozymase (*i. e.*, zymase freed from all activating substances) and coenzyme have been prepared with low fermenting power (since it seems to be impossible to free them completely from phos-

* In this connection, one must call attention to the terminology which Waldschmidt-Leitz has introduced. This worker, and his colleagues, have described a series of proteases, which they have studied and classified. According to their classification, the inactive or relatively inactive trypsinogen is called "trypsin," and the activated enzyme, ordinarily designated trypsin, is called "trypsin-kinase."

phate); upon addition of phosphate their activity is increased tremendously.

A nonspecific type of activation is postulated in the case of lipases by the Willstätter school.¹² In alkaline solution such widely different substances as egg albumin, bile salts and calcium soaps increase the activity of this enzyme. Their influence is ascribed to their simultaneous adsorption of enzyme and substrate.

Inactivation.—Enzymes are *poisoned* or inhibited by a great variety of substances. The fact that each enzyme has an optimum hydrogen ion concentration indicates that other acid ranges are relatively unfavorable; and in fact, in most instances there are sharp lines between activity and absolute inactivity. These may, in some cases, be due to an unfavorable action on the substrate rather than on the enzyme, but in others a destruction of the agent itself is brought about. Thus, the action of trypsin is definitely inhibited on both the acid and alkaline sides of its range of maximum rate of activity. This range is from about pH 7.5 to 9.5. At pH 6.5 and pH 11 there is a definite decrease in tryptic action.

As a rule, enzymes are fairly stable at reactions near their optimum pH, but this is not always the case. Thus trypsin, with an optimum at pH, is most stable at pH 2.3.¹⁵

The salts of the heavy metals have long been known to delay enzyme action. Minute traces of mercury, such as may be present in glass vessels, will inhibit urease markedly (and thus cause erroneous results) when this enzyme is used for the analytical determination of urea. Potassium cyanide combats this "poisonous" action of the heavy metals. Catechol and quinol in exceedingly minute amounts likewise inhibit the action of urease, but in this instance the cyanide has no antagonistic effect.²² Euler has shown that the inactivation of saccharase by mercury is reversible, the enzyme being recoverable by means of hydrogen sulfide. In general, substances which form insoluble salts with proteins inactivate enzymes. Besides heavy metals, this group includes picric and phosphotungstic acids.

Oxidizing agents are also injurious—not only strong oxidizers, but relatively weak ones, such as hydrogen peroxide and even atmospheric oxygen, which has recently been shown to be the primary cause of the inactivation of mussel catalase.²³ Many other compounds might be listed; some are "toxic" for certain enzymes and not for others; while a few, like the fluorides, are general enzyme inhibitors.

It is therefore evident that one must be careful, when selecting an antiseptic for enzyme work, that the agent will not affect the catalyst. Toluene is, in general, the most useful, since it has little activating or inactivating effect upon most enzymes. The fluorides and chloroform are usually to be avoided because of their inactivating action in many cases.

The inhibiting substances found normally (or pathologically) in biological fluids and tissues are known as *antienzymes*.²⁴ With few

exceptions they are not very specific. Normal blood serum, for example, contains antiproteases, as do also various animal tissues, egg white, intestinal mucus, and intestinal worms. Whether these inhibitors account for the fact that the gastro-intestinal mucosa is not digested, is still a moot question. Other living tissues, such as spleen and kidney, may be sewed into openings in the stomach, and as long as their blood supply is unimpaired, they suffer little digestion. It, therefore, seems likely that antienzymes are widely distributed, and that they are at least partly responsible for the nondigestion of living tissue. Among other factors are, probably, the buffers supplied by the blood, which prevent the reaction from becoming optimal for the enzyme.

Antitrypsin is not a true "immune" substance. It has been found that it does not follow the physicochemical laws of a true antigen-antibody reaction. The combination proceeds according to the law of mass action to form a dissociable inactive compound.

Many attempts have been made to cause the production of anti-enzymes by the parenteral administration of the corresponding enzymes. The results, however, have not been very decisive. With purified enzymes, it should now be possible to study this problem more carefully. A beginning has been made in the case of crystalline urease. If urease is injected into an animal it produces characteristic symptoms as a result of the conversion of urea into the toxic ammonium carbonate *in vivo*.²⁵ However, if the enzyme is administered in gradually increasing doses, it may be given in very large amounts without harm to the animal. This has been shown to be due to the production of antiurease.

Nature of Enzymes.—The Willstätter school has advanced the hypothesis that "enzymes are composed of one or more specific active groups, and of one more or less unspecific, and therefore changeable, colloid bearer; the former is considered responsible for the specificity and the latter primarily for the catalytic activity and for the stability of the active groups."¹² The colloid bearer, or "Träger," being changeable, may be substituted by other colloids, and this gives us the basis for the removal of the enzyme from its solution by alumina, kaolin, etc. Furthermore, it has led to the conception that the enzymes are not proteins, but that the colloid carriers, in the natural state, are proteins, and they may be replaced by other protein or nonprotein carriers. Although this hypothesis has formed the basis of a great number of splendid investigations, it is open to serious criticism.

It is asserted that the crystalline enzymes are merely crystals of globulins, or other proteins, which have adsorbed the enzyme in question. Any other crystalline protein, such as edestin, it is claimed, may be used to concentrate the enzyme.²⁶ However, this claim has been shown to be erroneous.²⁷ Furthermore, the statement that no enzymes are of protein nature is likewise unjustified. Several enzymes, such as rennin, urease and maltase,²⁸ are digested and hence inactivated by

proteolytic enzymes. Tauber²⁹ has shown that so-called "nonprotein enzyme solutions" may very easily have been too dilute to show even the most delicate protein tests while exhibiting a fair degree of enzyme activity.

However, one cannot say categorically that all enzymes are proteins. The ones mentioned above probably are. Trypsin which is not a typical protein has been isolated from an autolysate of hog's pancreas.³⁰ It is not crystalline and may be either a different enzyme from crystalline trypsin, or the same enzyme, freed from protein admixture. Emulsin is probably nonprotein, since it is not inactivated by pepsin, trypsin, or pancreatin. Since enzymes are colloids and these vary in their chemical composition, it is to be expected that enzymes will likewise vary in their chemical composition.

The Functions of Enzymes.—These catalysts may be shown to aid in nearly every physiologic function. Digestion is one succession of enzymatic reactions, going on under exceedingly favorable conditions of reaction, motility, and removal of products. Oxidations and reductions involve catalases, oxidases, dehydrogenases, besides such substances as glutathione and hematin. Other metabolic transformations require the aid of proteases, deaminases, lipases, and carbohydrases, too numerous to mention. The study of the blood is largely taken up with the various factors which go to form thrombin and modify its action. In almost every phase of biochemistry the enzymes play some part.

Autolysis^{24, 31} furnishes us with a method for the study of certain of these functions as well as of some less normal or even pathologic processes. Every animal tissue thus far examined will digest itself after removal from the body. The enzymes differ from those of the gastro-intestinal tract and therefore cannot be digestive enzymes which have been carried to the tissues by the blood stream. Proteases, lipases, carbohydrases, phosphatases, nucleinases are among the enzymes which have been shown to be active in autolytic mixtures. If bacterial growth is avoided (usually by the aid of toluene), autolytic digestion may continue for months, decreasing in rapidity as the products of digestion increase in concentration.

The rate of autolysis is considerably influenced by the reaction. At neutrality, or on the alkaline side, there is very little action; but as the mixture becomes acid, both the rate and extent of autolysis increase. The rise in the hydrogen ion concentration is due to the increase in amount of lactic and other organic acids at *postmortem*. According to Bradley, the proteolytic changes which occur are attributable to two groups of enzymes. The first acts at pH 7 to 2.6 and converts the acid tissue proteins into primary cleavage products. The second is active between pH 8 and 3 and attacks only the primary cleavage products of the proteins, breaking them down to amino acids.

It is seen that normal tissue is saved from autolysis by the preservation of its normal hydrogen ion concentration. If, however, acid

forms more rapidly than can normally be taken care of, the primary proteolytic action is favored and tissue breakdown occurs. The primary cleavage products are broken down further by the second enzyme which is effective in a slightly alkaline medium, and therefore can act even if the pH is brought back to normal. Thus are explained such physiologic phenomena as atrophy of the mammary gland after lactation, atrophy of the uterus after parturition, and generalized atrophy of old age. In all of these cases, a diminished blood supply tends to prevent adequate buffering of acids formed in the tissue metabolism. Pathologic degeneration, atrophies, the resolution of the exudate in lobar pneumonia, and much of postmortem change have been explained on a similar basis. If inhibitory substances are present in sufficient amount, autolysis will, of course, not occur. This is believed to be the chief reason why caseous tubercular material does not autolyze to an appreciable degree.

The reversibility of enzyme reactions has long been thought to offer a reasonable explanation for synthesis in the body—for food storage and tissue building. As stated above, synthesis has been demonstrated for several enzymes. In most cases, however, it is either very weak or is demonstrated under conditions which do not occur in the body. On the other hand, the conditions which actually obtain in the tissues cannot be reproduced *in vitro*. However, if the synthetic action of any particular enzyme is of importance, one would expect to find that enzyme present in large amount in those tissues which contain—or manufacture—large amounts of the substrate. On this basis, the mammary gland has been examined for the presence of lipase and lactase.³² Contrary to expectation, there is very little of the former enzyme and none of the latter present, and the syntheses of fat and lactose are still unsolved problems.

For the synthesis of bone, the action of phosphatase is believed to be one of a number of factors. This enzyme, present in the osteoblasts, the hypertrophic cartilage cells, and certain cells of the periosteum, hydrolyzes the salts of phosphoric esters brought to the ossifying zone by the blood, and thus causes a local increase in phosphate ions. A deposition of calcium phosphate is brought about in the vicinity of those cells.

Pathologically, enzymes may be of importance in several ways besides autolysis. It is known that the injection of enzymes into animals is almost invariably followed by marked symptoms. This may be due to the presence of protein. The only recent work with purified enzymes deals with the injection of crystalline urease and this causes a toxic effect as a result of ammonia poisoning;²⁵ but the evidence seems to indicate that urease is not the only enzyme capable of producing symptoms *per se*. If the enzymes normally present in certain body cells are toxic to other cells, we have a possible explanation for various obscure pathologic symptoms and conditions.

Catalase varies greatly in different tissues, and pathologically its

variations are even wider. It may be related to kidney and thyroid conditions. Lipases and esterases in blood seem to parallel the general health of the individual. More particularly, they are low in liver diseases and in diabetes, especially in diabetic lipemia. Fat necrosis is known to be caused by the action of pancreatic juice on fatty tissue in the abdominal cavity. Although the kidney and liver appear to be the organs richest in diastase, the greatest changes in blood diastase seem to occur in pancreatic disorders. It is increased if the pancreas is acutely inflamed or injured.

Myers and his coworkers have found that the high blood diastase of diabetic patients is reduced to normal by means of insulin.³³ In depancreatized dogs, on the other hand, a low blood diastase is found and this is raised to normal by insulin. In certain generalized diseases of bone, the plasma phosphatase is notably increased in concentration, and the increase in a general way parallels the severity of the disease.⁴ Scarcely any other condition produces such a result.

Enzymes are present in bacteria, yeasts, molds, etc., in great variety. Some of the harmful effects of pathogenic organisms are caused by products of enzyme activity; *e. g.*, the true bacterial toxins, which are substances synthesized by the organisms. Yeasts cause fermentation through the agency of zymase, and the proteolytic action of cheese molds is well known. Most life processes involve enzyme activity of one sort or another, and the healthy functioning of the organism depends largely upon the proper balancing of the various factors involved.

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REFERENCES

1. Bayliss, W. M.: *The Nature of Enzyme Action* (1914).
2. Willstätter, R.: *Problems and Methods in Enzyme Research* (1927).
3. Kleiner, I. S., and Tauber, H.: *J. Biol. Chem.*, **105**, 679 (1934).
4. Kay, H. D.: *Physiol. Rev.*, **12**, 384 (1932).
5. Haldane, J. B. S.: *Enzymes* (1930).
6. Tauber, H., and Kleiner, I. S.: *J. Biol. Chem.*, **96**, 745, 755 (1932).
7. Tauber, H., and Kleiner, I. S.: *J. Gen. Physiol.*, **15**, 155 (1931).
8. Mellanby, J., and Wooley, V. J.: *J. Physiol.*, **47**, 339 (1913); Northrop, J. N.: *J. Gen. Physiol.*, **16**, 323 (1932).
9. Hussey, R. G., and Thompson, W. R.: *J. Gen. Physiol.*, **9**, 217 (1925); Tauber, H.: *J. Biol. Chem.*, **87**, 625 (1930).
10. Falk, K. G.: *The Chemistry of Enzyme Actions* (1924).
11. Wasteneys, H., and Borsook, H.: *J. Biol. Chem.*, **62**, 15, 633, 675 (1924-25); **63**, 563 (1925).
12. Waldschmidt-Leitz, E.: *Enzyme Actions and Properties*, translated and extended by Walton (1929). Also *Annual Review of Biochemistry*, **1**, 69 (1932).
13. Sumner, J. B.: *J. Biol. Chem.*, **69**, 435 (1926); **70**, 97 (1926).
14. Northrop, J. H.: *J. Gen. Physiol.*, **13**, 739, 767 (1930).
15. Northrop, J. H., and Kunitz, M.: *J. Gen. Physiol.*, **16**, 267, 295, 313, 323, 339 (1932); **17**, 591 (1934).
16. Kunitz, M., and Northrop, J. N.: *Science*, **78**, 558 (1933).
17. Kober, P. A.: *J. Am. Chem. Soc.*, **39**, 944 (1917).
18. Kleiner, I. S., and Tauber, H.: *J. Biol. Chem.*, **106**, 501 (1934).
19. Lohmann, K.: *Biochem. Z.*, **254**, 332 (1932).
20. Harden, A.: *Ergebnisse d. Enzymforschung*, **1**, 113 (1932).
21. Myrback, K.: *Ergebnisse d. Enzymforschung*, **2**, 139 (1933).
22. Quastel, J. H.: *Biochem. J.*, **27**, 1116 (1933).

23. Marks, G. W., and Fox, D. L.: *J. Biol. Chem.*, **103**, 269 (1933).
24. Wells, H. G.: *Chemical Pathology* (1925).
25. Tauber, H., and Kleiner, I. S.: *J. Biol. Chem.*, **92**, 177 (1931); Kirk, J. S., and Sumner, J. B.: *J. Biol. Chem.*, **94**, 21 (1931).
26. Dyckerhoff, H., and Tewes, G.: *Z. physiol. Chem.*, **215**, 93 (1933).
27. Sumner, J. B.: *Proc. Soc. Exp. Biol. Med.*, **31**, 204 (1933); Northrop, J. H.: *J. Gen. Physiol.*, **17**, 165 (1933); Tauber, H., and Kleiner, I. S.: *J. Biol. Chem.*, **104**, 259 (1934).
28. Tauber, H., and Kleiner, I. S.: *J. Gen. Physiol.*, **16**, 767 (1933).
29. Tauber, H.: *J. Biol. Chem.*, **99**, 257 (1932).
30. Tauber, H., and Kleiner, I. S.: *J. Biol. Chem.*, **104**, 267 (1934).
31. Bradley, H. C.: *Physiol. Rev.*, **2**, 415 (1922).
32. Kleiner, I. S., and Tauber, H.: *J. Biol. Chem.*, **99**, 241 (1932).
33. Myers, V. C., and Reid, E.: *J. Biol. Chem.*, **99**, 595, 607 (1933); Reid, E., Quigley, J. P., and Myers, V. C.: *Ibid.*, **99**, 615 (1933).

CHAPTER XI

DIGESTION

VERY few foods are ready for utilization in the state in which they are ingested. Water, glucose and the inorganic salts constitute this small group, to which may be added the greater part of the vitamins, and perhaps a portion of the fat and other lipoids. The remainder must undergo more or less disintegration to permit them to be absorbed and metabolized. Cooking usually renders food more easily digested. It adds to the flavor and thus reflexly stimulates the flow of saliva and gastric juice. It causes partial hydrolysis of some of the food-stuffs; collagen is converted to some extent into gelatin, starch is rendered more digestible, etc.

It was formerly thought that some cooked foods were less digestible than raw. Hard-boiled eggs, for instance, were considered to be less easily handled than soft-boiled, and raw eggs were frequently administered to invalids. This has been shown¹ to be incorrect. Cooking also destroys or inhibits the growth of bacteria, yeasts, and parasites which might otherwise gain access to, and flourish in the gastro-intestinal canal. Another factor is the subdivision of food, accomplished chiefly by mastication. As would be expected, this tends to accelerate the digestive action by increasing the surface of the substrate. William Beaumont beautifully demonstrated the importance of mastication in his classic experiments on Alexis St. Martin.

The chief agents of digestion, however, are the enzymes. Acting at favorable, if not optimum, hydrogen ion concentrations and temperatures, and influenced by peristalsis and the rapid removal of products, these catalysts effect far-reaching transformations.

SALIVA

Each fluid which enters the gastro-intestinal tract is more or less complex and is admirably adapted to its functions in digestion, physically as well as chemically. Ordinarily saliva flows from the various salivary glands at a rate sufficient to keep the mouth and esophagus comfortably moist. When food is to be eaten, the sight, odor, or even the thought of it will initiate an increased salivary flow. This fluid, since it is about 99.4 per cent water, is well suited to moisten foods and dissolve quickly the water-soluble taste-producing ingredients. Taste is an additional factor in the reflex stimulation of the flow of the digestive fluids, and in this way the high water content of saliva serves to aid in continuing the secretions during the period necessary for them. The *mucin* and other proteins give it the viscosity, the lubricating quality useful in deglutition. Its digestive function is dependent upon the salivary amylase, often called *ptyalin*.

Among other constituents which have been demonstrated are urea, uric acid, inorganic salts, and various enzymes. The urea, uric acid, and some of the salts are probably "accidental" constituents; that is, they are present in blood and find their way into the saliva by osmosis and diffusion. This explanation may, however, not be tenable since the osmotic pressure of saliva differs considerably from that of blood, and certain of the inorganic ions (K^+ , Ca^{++} , HCO_3^-) are at higher concentrations in parotid saliva, and some (Na^+ , Cl^-) are at lower concentrations. This indicates, of course, that saliva is not a simple transudate or dialysate.² The small amount of salts present has a distinct function, since the amylase (ptyalin) requires some negative ion as an activator. The following are effective as coenzymes in descending order:

(a) Cl^- , Br^- ; (b) I^- , NO_3^- ; (c) SO_4^{--} , PO_4^{---} , CH_3-COO^- .

Amino acids also increase its activity.

Just what rôle potassium sulfocyanide plays is still a problem. The old idea of its parallelism with tobacco smoking cannot be accepted. In the absence of sodium chloride and at the pH of saliva, it increases the amylase activity and hence it may possibly be a "facultative" coenzyme.³ The reaction of human saliva usually ranges from pH 6.2 to 7.6.⁴ The salivary pH varies in a rather definite manner throughout the day. It rises sharply just after meals, but quickly falls to a point slightly lower than that occurring just prior to meals. Between meals it approaches neutrality. The other enzymes present may include catalase, maltase, lipase, urease, and a protease. The source or importance, if any, of these is at present a matter of speculation. Unless saliva is obtained by cannulation of a duct, the presence of bacteria, food residues, desquamated epithelia, and leukocytes must all be considered in studying these "trace" enzymes. No amylase is present in the saliva of the sheep, goat, dog or cat.⁵ It is found in the guinea-pig, rat, mouse, rabbit, and in man. In man it occurs in saliva even at birth and increases in concentration with age.

The salivary amylase has an optimum pH of 6.5, but it has a wide range of activity (pH 4-9). It therefore continues its action in the stomach after being swallowed with its substrate and stops only when the acidity becomes greater than pH 4. This frequently takes an appreciable length of time, since the gastric juice must permeate a mass of material of variable size and buffering power. Once acidified, its activity cannot be restored by neutralization.⁵ The presence of starch is said to protect ptyalin from the destructive action of the hydrochloric acid even at a concentration of 0.16 per cent.⁶ *In vitro*, saliva loses its amylolytic power rather rapidly, even though it is said to contain some unidentified colloid (named by Ege "ptyalin stabilizer"⁷) which is more effective in maintaining amylolytic activity than sodium chloride.

Ptyalin acts readily on cooked starch and slightly on raw starch.

Glycogen is hydrolyzed by the salivary amylase of omnivora, including man, but not by that of herbivora.⁸ In the course of starch hydrolysis, a series of polysaccharides are formed, of progressively smaller molecular size, thus: Starch \rightarrow soluble starch \rightarrow erythrodextrin \rightarrow α -achroodextrin \rightarrow β -achroodextrin \rightarrow maltose. Maltose may be demonstrated to be present very early in the digestion of starch and continues to increase in amount. For this reason it is assumed that the larger molecules are reduced in size gradually by the splitting off of molecules of maltose. However, the exact course of this hydrolysis is by no means clear.

Since food remains in the mouth only a short time, the chief site of salivary digestion is in the stomach. The duration and extent of this amylolytic digestion is, of course, variable. Cameron⁹ states that, within fifteen or twenty minutes of ingestion, about 75 per cent of the starch of mashed potatoes and 60 per cent of that of bread may be completely changed to maltose, and part of the remainder is partially digested. The question of salivary maltase activity has not been definitely settled. Such an enzyme has generally been considered present. It is probable that it varies in amount with the time of day and with the individual, and these variations may account for the occasional negative reports which have appeared. However, in this connection, the possibility of inversion by salivary bacteria must not be overlooked.

GASTRIC JUICE

The secretion of gastric juice may be stimulated mechanically. This was first shown by Beaumont and has again been demonstrated by Ivy and his coworkers. The mechanical factor, however, is probably not as important as the psychical and humoral. According to Pavlov, the amount of juice secreted is proportional to the quantity and character of the food received by the stomach. Some foods, such as raw meat, can excite gastric secretion on local contact with the gastric mucosa, while other foods only do so after they have been digested.¹⁰ Hydrolyzed proteins act chiefly after they have reached the intestine.

Among the secretagogues for gastric juice may be mentioned certain of the amino acids, various amines (including histamine), soap, dilute glycerol, spinach juice, MgSO_4 and saponin. Some of these are effective via the stomach, others only via the intestines, while some, such as histamine and β -alanine, act by way of both routes. Secretagogues are more potent when applied to the gastric mucosa than when introduced into the blood stream. In the former case, they are not absorbed but act either by exciting the formation of a hormone or by a local nervous action, or both. Whether *gastrin*, a hormone supposed to be present in gastric and intestinal mucosa extracts, really exists, is questioned.

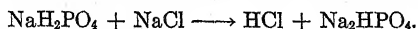
Gastric Digestion.—In gastric juice there are present *pepsin*, a *lipase*, and *hydrochloric acid*. *Rennin* is found in infancy but de-

creases up to maturity; the reverse is true of pepsin. The infant's gastric digestion is very incomplete, the casein being precipitated as calcium paracaseinate, which is subsequently more thoroughly digested in the small intestine. The contents of the infant's stomach are emptied very quickly, usually before complete acidification has taken place. Thus a baby can take at a feeding more fluid than the capacity of the stomach.¹¹

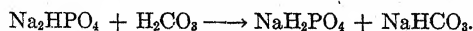
The evacuation of the stomach undoubtedly does not depend upon the acidity of the chyme, as formerly believed. Beaumont showed that various types of foods required different periods for digestion. This is particularly noticeable in the case of fats, which remain longest in the stomach and retard the digestion of other foods. Carbohydrates are retained for the shortest length of time while proteins are somewhat intermediate. The retarding action of fats has been shown by Ivy and Farrell¹⁰ to be partly humoral in character, but whether the agent is a chalone (inhibitory autacoid) or some constituent of the fat is undecided. The gastric evacuation time for various articles of diet has been determined by Hawk, Rehfuess, and Bergeim¹² by fractional analysis of gastric contents. It is fairly characteristic for individual foods, for most of which it averages two to three hours.

Gastric Acidity.—Pavlov stated in 1910 that "the gastric juice as it flows from the glands possesses a constant acidity."¹³ It appears certain that pure gastric juice, uncontaminated by food, mucus, bile, etc., has a constant acidity—at least normally. Hollander and Cowgill¹⁴ have demonstrated that Pavlov pouch dogs can yield such juice if the technic is designed to avoid contamination with mucus, the secretion of which may be provoked by mechanical stimulation of the mucosa. The pH of this pure gastric juice of maximum and constant acidity was found to be 0.91 ± 0.02 . That is, "the parietal secretion is essentially an isotonic solution of hydrochloric acid" of 0.15 *N* concentration; or about 0.6 per cent HCl. It is probable that the acidity is identical, or nearly so, in cats, dogs and humans.

The formation of a free mineral acid of relatively high concentration from a biological standpoint, in a tissue the cells of which are alkaline is a most interesting problem. It has been the subject of a number of investigations and has led to several hypotheses,^{15, 16} only a few of which we may mention. The reaction of a chloride with an acid phosphate may be considered as one possibility; thus:



As the HCl is secreted, the Na_2HPO_4 may react with carbonic acid:



The bicarbonate is returned to the blood and the acid phosphate is ready to react again.

The hypothesis of Mathews postulates that ammonium chloride is secreted and hydrolyzed while still in the lumen of the gland into HCl

and NH_3 . The NH_3 or NH_4OH is absorbed by the cells, permitting the extrusion of the HCl . The high concentration of ammonium salts in the gastric mucosa is an argument in favor of this view.

Hanke has found in aqueous extracts of the gastric mucosa an enzyme which can cause the hydrolysis of organic chlorides, thus liberating acid. Such organic chlorides are present in the gastric tissue. However, not all of the acid formed is hydrochloric acid and the enzyme is distributed in other tissues of the body.

It is possible that a physicochemical process is involved in this mechanism—some membrane effect of a nature similar to that described by Donnan.

Although the hydrochloric acid is secreted at a relatively high concentration, gastric juice, as ordinarily obtained by stomach tube, or fistula, is much less acid. Boldyreff claims that intestinal regurgitation is responsible for most of the neutralization. This, however, is contested by various authorities. Golob¹⁷ states that the presence of appreciable amounts of bile in gastric contents after a test meal is abnormal. McCann¹⁸ eliminated the duodenal juices surgically by shunting them away from the pylorus into the distal portion of the ileum. In such animals fractional gastric analysis showed no variations from the curves obtained before the operation. Undoubtedly mucin neutralizes some of the hydrochloric acid and the mucus secretion of the antrum is particularly alkaline in character.

A constant basal amount of mucus, indicated by the work of Ivy, is sufficient to combine with all of the acid present in the resting stage. Although the secretion of mucin increases as the hydrochloric acid secretion continues, it soon reaches a maximum which is far from sufficient to combine with all of the acid. In addition to the gastric mucin factor and the possible occurrence of some duodenal regurgitation, there is also the neutralizing and buffering power of saliva and of foods. All of these tend to prevent the gastric contents from attaining the maximum acidity of the gastric juice and account for the fact that the contents after a test meal are more nearly equivalent to 0.2 per cent than to 0.5 per cent hydrochloric acid.

The presence of this mineral acid is advantageous from several standpoints. Besides converting *pepsinogen* (the inactive pepsin) or, in the case of the young, *prorennin*, into the active stage, and providing pepsin with a favorable hydrogen ion concentration, it causes some proteins to swell. This of course, allows the substrate to be more easily digested. The antiseptic power of hydrochloric acid has been disputed, but it is undoubtedly of some importance. Alvarez says, "contrary to popular belief, there is rarely any fermentation in the stomach. Its contents are too acid and the food does not remain long enough for gases to be formed."¹⁹ However, even normally, gastric contents are not acid enough for a sufficient length of time to sterilize the chyme completely, and the intestinal contents nearly always contain living micro-organisms.

Pepsin, derived from pepsinogen by the action of the acid, is the chief enzyme present in gastric juice. Its action upon native proteins in acid media (the optimum *pH* varies with the substrate) is extremely rapid and powerful, the large molecules being split to proteoses and peptones. However, we cannot definitely say what linkages pepsin attacks. The products are relatively large fragments, the free amino acids being produced in exceedingly small amounts. Furthermore, since no known simple peptides have been split by pepsin, many biochemists believe that the point of attack cannot be a peptide linkage. Waldschmidt-Leitz, however, found that in the course of peptic digestion, —COOH and —NH_2 groups are usually set free in equivalent amounts. This has been corroborated by Bradley who concludes that "the weight of evidence, therefore, appears to us to be definitely in favor of the view that pepsin opens specific peptide links."¹⁶

Gastric lipase is present only in small amounts. Its action is therefore very slight. The statement commonly made that gastric lipase acts only upon emulsified fats probably has as a foundation the fact that fat is not easily emulsified in the acid gastric medium, and therefore the enzyme must necessarily exert its action principally upon preformed emulsions. That a slight fat hydrolysis in the stomach will be demonstrated to have some physiologic value is not improbable.

The fact that rennin is an enzyme separate and distinct from pepsin, has been shown in the preceding chapter. It should be remembered, however, that rennin is present only during early life. It splits the casein complex yielding some peptone and *paracasein*, which, combining with calcium, forms the curdy precipitate, calcium paracasein, or calcium paracaseinate. This action undoubtedly retards the passage of this important milk protein through the intestinal canal and permits of a more complete digestive action in the duodenum. In the adult no rennin is present nor is it necessary. The hydrochloric acid alone can precipitate casein, and not only can pepsin precipitate it but it can, of course, digest it to proteoses and peptones.

Cannon's theory of the acid regulation of the emptying of the stomach was dominant during the early part of this century. According to this hypothesis, free hydrochloric acid when in sufficient strength stimulates the opening of the pylorus from the gastric side, and causes it to close when the duodenal mucosa is acidified. Working along varied lines, a number of investigators have shown this conception to be erroneous.²⁰ The most recent work¹⁸ indicates that the stomach begins to empty very soon after a meal, at first slowly, but with increasing rapidity as digestion progresses. This is independent of the acidity. The fundus, containing the bulk of the digesting mass, remains almost motionless, but its muscular wall exerts a tonic pressure upon the digesting food. As digestion proceeds, the pyloric end of the stomach relaxes and its peristaltic activity diminishes. The mechanism for the control of the sphincter does not appear to be a specific stimulus for relaxation either by hydrochloric acid or by digestive products.

The irritability of the antrum and, in McCann's experiments, the presence of raw proteins, seem to control the activity of the antrum. The change to a fluid state also seems to facilitate the emptying of the stomach.

DIGESTION IN THE SMALL INTESTINE

The acid chyme, ejected from the stomach into the duodenum, is now subjected to the influence of three different secretions: Intestinal juice, bile, and pancreatic juice.

Pancreatic secretion is evoked by many substances,¹⁰ but the specific physiologic excitant is *secretin*. This hormone is probably present in the intestinal mucosa as *prosecretin*. The effect of the hydrochloric acid of the chyme is to change prosecretin into secretin, which then goes by way of the blood circulation to the pancreas, causing this gland to secrete.

The *secretion of intestinal juice* is chiefly influenced by local stimulation (mechanical or chemical). However, there is some evidence that a hormonal or humoral type of stimulus is also operative.¹⁰ Possibly secretin is the hormone.

As regards *bile secretion*, secretin again seems to be involved; although Mellanby has brought forward evidence to indicate that the secretin stimulation of bile formation is only indirect. That is, secretin stimulates the pancreas and the metabolic products of the pancreas are the biliary excitants. At best, however, the action of secretin is slight; and a number of digestive products have been found to exert similar action. Bile salts, however, exert marked action and are undoubtedly the best cholagogues. The secretion of bile by the liver is continuous, but its passage into the intestine is intermittent. The mechanism of bile flow into the intestine is in dispute. Either the contraction of the sphincter of Oddi or the tonus of the duodenum prevents the bile from flowing continuously into the small gut. Its passage into the gallbladder is unimpeded, and here it is concentrated by the specific activity of the gallbladder's mucosa. This may amount to a tenfold concentration of "liver bile."

During periods of digestion (particularly during fat digestion) the sphincter of Oddi relaxes or the tonus of the small intestine is lowered, and bile is forced into the intestine. Burget²¹ asserts that the gallbladder plays only a passive rôle in this process; that it is "regulated by tonicity and peristalsis of the duodenum, with elasticity and intra-abdominal pressure as auxiliary factors." If the gallbladder is incapable of effective contractions, as Burget maintains, the theory of a hormonal origin of gallbladder contractions¹⁰ would seem to be untenable.

Functions of the Bile.—The *bile pigments*, *bilirubin* and *biliverdin*, are derived almost entirely from hemoglobin, and are considered to be excretory products resulting from the disintegration of its pigment complex. Cholesterol and other lipoids, also found in the bile, are

likewise excretory, or chiefly so. A miscellaneous group of substances is also excreted, such as various inorganic salts, drugs, dyes, and toxins.

The most important physiologic functions of bile are due to the presence of the *bile salts*. These are the sodium and potassium salts of *glycocholic* and *taurocholic* acids. Glycocholic acid yields glycocholate and cholic acid on hydrolysis, and taurocholic acid splits up to give taurine and cholic acid. Taurine is structurally related to cysteine, and cholic acid to cholesterol; but whether this indicates their derivation in the body is not known.

As stated before, the bile salts are cholagogues and apparently are absorbed from the intestine and resecreted in the bile time after time. They have a dual effect upon *fat digestion*. By lowering surface tension, they aid in the emulsification of the fats. This permits more rapid fat digestion by the pancreatic lipase and they may allow fats and other lipoids to be absorbed undigested. Emulsification is also aided by the soluble proteins present in the pancreatic juice, the *succus entericus*, and perhaps in the chyme. The second effect of the bile salts is their direct influence upon the absorption of the fatty acid fraction of the digested fats. We can no longer accept the view that they are absorbed as soaps.

Verzar and Kúthy²² point out that the alkaline soaps can be in solution only at pH 9, an alkalinity much greater than is found in the intestine. They show that the fatty acids, finely emulsified, form diffusible solutions with the paired salts of sodium taurocholate and glycocholate. These solutions are formed in neutral or slightly acid media and have a low surface tension. They postulate the formation of fatty acid—bile acid complexes. These are taken up from the intestinal lumen and are assumed to be hydrolyzed in the intestinal wall. The fatty acids are absorbed and the bile acids remain in the intestine ready to combine with more fatty acids. These investigators have found bile acids in the intestinal mucosa hours after their administration, while none was left in the lumen of the intestine. This explains how the comparatively small amount of bile salts, normally present, can convert all the fatty acid ordinarily present into a diffusible and absorbable state. Drummond²³ is of the opinion that this constitutes the most important function of bile and may even be operative in the case of hydrocarbons which have been found to be absorbed in small but definite amounts.

Bile also accelerates the action of the pancreatic lipase²⁴ and this too is undoubtedly due to the bile salts. The mechanism of this acceleration has not been worked out. The question arises, however, whether this may not be a result of the formation of bile salt-fatty acid combination, thus accelerating the reaction by removing a product of the reaction. Other lipases and esterases, however, are inhibited by bile; *e. g.*, castor bean lipase and hepatic esterase. The pancreatic amylase is also said to be accelerated by bile.

The bile, furthermore, is one of the three fluids which constitute

factors in neutralizing the hydrochloric acid of gastric juice. McClen-don found the pH of the lower duodenum in humans to vary from 4.5 to 5.1 and that of the ileum from 5.9 to 6.5. Okada's observations range from 4.8 to 7.9. These acidities of the ileum and jejunum are due to the presence of weak acids, not to hydrochloric acid. In bile it is the sodium bicarbonate which contributes most of the alkalinity; the earlier view that the bile salts were the most important factors is evidently erroneous. Schmidt is of the opinion that the neutralizing influence of bile is secondary to that of the pancreatic and intestinal juices. (See also Chapter IV.)

Pancreatic Juice.—Pancreatic juice is undoubtedly the most important digestive fluid in the body, containing a powerful amylase, lipase and protease. It is distinctly alkaline, with a pH of about 8. The pancreatic enzymes include *amyllopsin*, *steapsin*, *trypsinogen*, *erepsin*, and some disaccharide-splitting enzymes. There is also said to be rennin present, but since trypsin has been shown to have a rennet action if conditions are suitable,²⁵ it is questionable whether there is a separate milk-clotting enzyme in pancreatic juice.

The amylase, *amyllopsin*, exerts an action upon starches very similar to that of the salivary amylase, but its optimum pH is 7, while ptyalin acts best in faintly acid reaction. The final product is maltose. Glucose may also be discovered among the digestion products, probably as a result of the activity of a maltase. *Steapsin*, a lipase, or fat-splitting enzyme, is a relatively weak enzyme when pure pancreatic juice is tested. It is rendered quite powerful by "nonspecific" activation by the bile salts; that is, this activation, or acceleration, can be brought about by various substances including the bile salts which are believed to adsorb both the enzyme and substrate and thus facilitate the reaction. The optimum pH is 8. The lipolytic action is, of course, also influenced by the degree of emulsification of the fat and by the rapidity of removal of the products. In both of these processes bile has a most important part. It is therefore quite evident why an animal, deprived of bile, should have fatty stools in spite of the continued presence of the most active lipase in the body. There seems to be little difference in the digestibility of the different fats, except that the ones with lower melting points are slightly better assimilated than those with higher melting points;²⁶ which may be related to the relative facility of emulsification.

Pancreatic juice, as obtained directly from the pancreatic duct, has weak proteolytic powers. This was first ascertained by Claude Bernard. Later it was shown that a very powerful protease could be obtained from the pancreas or from its juice by various processes; but it was not until 1900 that *enterokinase* was discovered in the intestinal mucosa. The inactive, or slightly active protease, *trypsinogen* has specific proteolytic powers.²⁷ It acts upon peptones and protamines, but not upon casein, gelatin, fibrin or proteins of higher molecular weights. In the ordinary course of events, the pancreatic juice, con-

taining trypsinogen, is poured into the intestinal tract, and there the zymogen (trypsinogen) is activated by enterokinase, a constituent of the intestinal juice. The resulting enzyme, *trypsin*, is capable of hydrolyzing the complex proteins, and also the products of peptic digestion, to the peptide stage. Its optimum pH is, in general, about 8, although this varies somewhat depending upon the protein used as substrate.

Pancreatic juice also contains an *erepsin* (a proteolytic enzyme), similar to the intestinal erepsin. In fact, Waldschmidt-Leitz and his coworkers believe that the two are identical.²⁷ According to him, erepsin is elaborated by the pancreas and is introduced into the intestinal canal along with the other constituents of pancreatic juice. It is then taken up by the intestinal mucosa and subsequently secreted in the intestinal juice. The early view that this enzyme attacks prolamines, histones, and casein is incorrect, according to the same school; nor does it hydrolyze peptones, but only peptides. Erepsin is really a mixture of peptidases which split the polypeptides to amino acids. Its optimum pH is 7.8.

Activated pancreatic juice is thus capable of digesting proteins to the amino acid stage, carbohydrates to the disaccharides and, in some cases, to the monosaccharides, and fats to their constituent parts.

Intestinal Juice.—The *succus entericus*, or intestinal juice, is an alkaline secretion (pH 7.7) produced chiefly by the mucosa of the duodenum, but also, in lesser amount, by the lower portions of the small intestine. It contains many enzymes of a diversified nature. Its carbohydrases include an amylase and, what are more important, disaccharide-splitting agents, *maltase*, *sucrase*, and *lactase*. Lactase is found in much greater abundance in the intestine of the young mammal than in that of the adult. There is a lipase, several proteases, and enzymes which break down the nucleic acids. The proteases include the constituents of erepsin and, possibly, a rennin, although this point requires reinvestigation.

Nucleoproteins undergo primary cleavage in the stomach as a result of peptic action, or in the intestine, because of the action of trypsin. The resulting nucleic acid is broken down to its constituent nucleotides by the nucleinase of the *succus entericus*. Nucleotidases then act upon the nucleotides, forming phosphoric acid and nucleosides. Apparently only those nucleotidases which attack the purine nucleotides are found in the intestinal juice. Those which attack the analogous pyrimidine compounds occur in the intestinal mucosa, not in its secretion. In the mucosa there are also found additional purine nucleotidases and a purine nucleosidase. No pyrimidine nucleosidase has been found.²⁸

The intestinal juice also contains *enterokinase*, which transforms the feebly active trypsinogen of the pancreatic juice into the powerful trypsin.

In addition to the highly active intestinal juice, which is controlled by nervous and hormonal influences, there is a periodic se-

cretion of the mucosa. This is secreted about every two hours, even during starvation, and contains chiefly mucin and excretory products.

It is interesting to note that the body is well provided with factors of safety from a digestive viewpoint. The surgical removal of the stomach is an everyday occurrence. With a little care as to diet, gastrectomized individuals digest all the primary foodstuffs and may live for many years. Similarly, depancreatized animals survive for months or even years, provided they are supplied with insulin to make up for their endocrine deficiency. It is evident that, under these conditions, the variety of enzymes which remain operative can produce a sufficient quantity of the digestive products to maintain life.

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REFERENCES

1. Bateman, W. G.: *J. Biol. Chem.*, **26**, 263 (1916); Rose, M. S., and MacLeod, G.: *J. Biol. Chem.*, **50**, 83 (1922).
2. Beer, de, E. J., and Wilson, D. W.: *J. Biol. Chem.*, **95**, 671 (1932).
3. Miller, L. P.: Contributions from the Boyce Thompson Inst., **3**, 287 (1931).
4. Henderson, M., and Millet, J. A. P.: *J. Biol. Chem.*, **75**, 559 (1927).
5. Schwarz, C., et al.: *Fermentforschung*, **9**, 50, 57 (1926).
6. Maestrini, D.: *Atti accad. Lincei* (v), **29**, II, 291 (1920).
7. Ege, R.: *Beret. 18th Skand. Naturforskem/de*, **1929**, 266.
8. Lücking, W.: *Deutsch. tierärztl. Wchschr.*, **34**, 257 (1926).
9. Cameron, A. T.: *A Text-book of Biochemistry* (1933).
10. Ivy, A. C.: *Physiol. Rev.*, **10**, 282 (1930).
11. McLean, S., and Fales, H. L.: *Scientific Nutrition in Infancy and Early Childhood* (1925).
12. Hawk, P. B., Rehfuess, M. E., and Bergeim, O.: *Am. J. Med. Sci.*, **171**, 359 (1926).
13. Pavlov, I. P.: *The Work of the Digestive Glands* (1910).
14. Hollander, F., and Cowgill, G. R.: *J. Biol. Chem.*, **91**, 151 (1931); Hollander, F.: *J. Biol. Chem.*, **97**, 585 (1932); **104**, 33 (1934).
15. Hollander, F.: *J. Am. Inst. Homeop.*, **22**, 311 (1929).
16. Bradley, H. C.: *Yale J. Biol. Med.*, **4**, 399 (1932).
17. Golob, M.: *Am. Med.*, **22**, 281 (1927).
18. McCann, J. C.: *Am. J. Physiol.*, **89**, 483, 497 (1929).
19. Alvarez, W. G.: *Nervous Indigestion* (1930).
20. Murlin, J. R.: *J. Nutrition*, **2**, 311 (1930).
21. Burget, G. E.: *Am. J. Physiol.*, **81**, 422 (1927).
22. Verzar, F., and Küthy, A.: *Biochem. Z.*, **205**, 369 (1929); **210**, 265, 281 (1929); **230**, 451 (1931).
23. Drummond, J. C.: *Harvey Lectures*, **28**, 202 (1934).
24. Schmidt, C. L. A.: *Physiol. Rev.*, **7**, 129 (1927).
25. Tauber, H., and Kleiner, I. S.: *J. Biol. Chem.*, **104**, 271 (1934).
26. Langworthy, C. F., and Holmes, A. D.: U. S. Dept. Agric., *Bull.* **310** (1915); Holmes, A. D., and Deuel, H. J., Jr.: *J. Biol. Chem.*, **41**, 227 (1920).
27. Waldschmidt-Leitz, E.: *Enzyme Actions and Properties* (1929).
28. Bodansky, M.: *Introduction to Physiological Chemistry* (1934).

CHAPTER XII

THE BIOCHEMISTRY OF BACTERIA, YEASTS AND MOLDS

ALTHOUGH the production of alcohol by fermentation is probably the oldest of the chemical arts, it was only in 1870 that Pasteur showed that yeasts and bacteria were the causal agents of many well-known processes of fermentation and putrefaction, while it was left to Wehmer in 1891 to begin the first classical experiments on the biochemistry of the lower fungi or molds. Since this time, however, the metabolism of micro-organisms has been widely studied and the results have been collected in many biochemical monographs.^{1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 23, 31} The present article cannot hope to discuss the whole of this work, but deals only with representative types of reactions brought about by species of bacteria, yeasts and molds under varying conditions.

METABOLISM OF BACTERIA

This section will be divided into two parts dealing with the action of bacteria on nonnitrogenous and nitrogenous substrates.

I. ACTION OF BACTERIA ON NONNITROGENOUS SUBSTRATES

(a) **Production of Hydrogen from Carbohydrates Including Cellulose.**—The production of hydrogen by the bacterial fermentation of sugars and of hydrogen frequently accompanied by methane by the fermentation of cellulose was early recognized. It was concluded that the occurrence of hydrogen and methane in bogs, river mud and manure heaps was due to the breakdown of cellulose contained therein; while the production of hydrogen and methane in the intestine of the ox was also due to the decomposition of cellulose by an "organized ferment" (micro-organism).

It has also become evident gradually that of the schizomycetes which produce gas from sugars, only members of the genera bacterium and clostridium produce hydrogen. This was confirmed in the quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of bacteria.¹³ Returning to the fermentation of cellulose, Omelianski using an inorganic medium and inoculation from mud and manure heaps, claimed to have isolated pure cultures of two spore-bearing anaerobes, both attacking cellulose, one with the production of hydrogen (*B. fossicularum*) and the other of methane (*B. methanigenes*). Kellerman and McBeth, however, claim to have isolated from the hydrogen-producing culture two cellulose-attacking aerobes and five contaminants. Khouvine isolated in pure culture from the human intestine a strict anaerobe, *B.*

cellulosae dissolvens, which will not develop on glucose or cellobiose but attacks cellulose, producing butyric acid, ethyl alcohol, CO_2 , H_2 and a pigment. The formation of hydrogen from pectin was accomplished using the anaerobe *Plectridium pectinovorum* Störmer and from starch, using bacteria of the amylobacter group. The formation of hydrogen (and often methane) from cellulose by aerobic and anaerobic thermophilic organisms has been described in many other papers.¹⁴

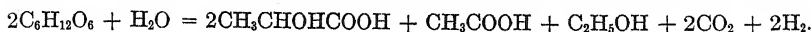
Using thermophilic organisms, Raistrick and his collaborators made the interesting observation that when glucose was added to a cotton cellulose fermentation while it was vigorously producing almost pure methane ($+\text{CO}_2$) the methane was completely replaced by hydrogen. Similar results in sludge fermentations are reported by Boruff and Buswell. Whereas cellulose was decomposed almost quantitatively according to the equation $\text{C}_6\text{H}_{10}\text{O}_5 + \text{H}_2\text{O} = 3\text{CO}_2 + 3\text{CH}_4$, the simpler carbohydrates (starches and sugars) gave much less total gas and the gas was composed chiefly of CO_2 and H_2 , little or no CH_4 being found.

It appears, therefore, that in the production of methane, cellulose does not ferment by way of the ordinary glucose molecule. On the other hand, several authors did isolate glucose from cellulose fermentations by adding reagents which inhibited the growth of the organisms without inhibiting their enzymic activity.

(b) **Production of Methane from Cellulose and from the Calcium Salts of Fatty Acids.**—As shown above, the production of methane as well as hydrogen frequently occurs in cellulose fermentation. Hoppe Seyler obtained in slow fermentations only $\text{CO}_2 + \text{CH}_4$ and concluded that acetic acid was an intermediate product. This view was supported by the production of CH_4 from calcium acetate, from calcium formate and butyrate, and from the propionate, gluconate and lactate, while Neave and Buswell described in sludge fermentations the quantitative conversion of fatty acids into $\text{CO}_2 + \text{CH}_4$. It seems probable, therefore, that the production of CH_4 from cellulose is due to the secondary fermentation of fatty acids. Raistrick, in fermenting cotton with a thermophilic culture, found that during the first three days $\text{CH}_4 + \text{CO}_2 +$ calcium acetate were produced while after three weeks a secondary fermentation of the acetate occurred, yielding more $\text{CH}_4 + \text{CO}_2$. In the early fermentation almost pure CH_4 was obtained and the process was worked on the large scale. A number of attempts have been made to produce combustible gases on the large scale, particularly by sludge fermentations using vegetable matter and municipal wastes, the latter consisting of 25 per cent each of ash, protein, fat and carbohydrate (almost exclusively cellulose). Imhoff equipped the sludge reduction tanks at Essen with gas collectors and connected them to the city mains. The gas was satisfactory for municipal use. Buswell did the same at Decatur, Ill., and was producing 200,000 cubic feet of gas per day, due to considerable wastes from starch works being discharged into the city drainage system. With

open sludge digestion tanks, CH_4 formed only 20 per cent, but with closed tanks 60–70 per cent of the total gas. The total amount of gas that can be produced from municipal wastes is, however, small and amounts to about one twenty-fifth of the total required for the general use of the community.

(c) **Production of Formic Acid and its Degradation to $\text{H}_2 + \text{CO}_2$.**—Harden followed quantitatively the breakdown of sugar by *Bact. coli communis* and constructed the following equation from the results:



Acetic acid and ethyl alcohol were present in equimolecular proportions suggesting their origin from a common parent presumably acetaldehyde. Grey showed aldehyde was present and Neuberg and Nord confirmed this by use of the sulfite method. The immediate precursor of CO_2 was shown to be formic acid. Pakes and Jollyman found that *Bact. enteritidis* grown in 1 per cent formate broth cultures gave $\text{H}_2 : \text{CO}_2$ in the ratio of 1:1. The same behavior was exhibited by a large number of the bacteria which evolve gas from glucose, *e. g.*, *Bact. coli*. Bacteria which, when grown in glucose solutions, do not produce gas, *e. g.*, *Bact. typhosum*, were incapable of giving gas from formate. Yeasts which give no free H_2 from sugar do not give hydrogen from formate. This correlation between bacterial gas production and formate decomposition was extended by Penfold who succeeded in producing a modified strain of *Bact. coli* which yielded acid but no gas from glucose, fructose, mannose, lactose and dextrin, but still produced gas from mannitol, glycerol, sorbitol and from formates. Harden and Penfold showed that this modified strain gave less $\text{H}_2 + \text{CO}_2$ but more formic acid. The power to decompose formates was therefore impaired but not destroyed. Grey using Penfold's method obtained a strain of *Bact. coli* which gave no gas with sugars, alcohols or formates. Harden's figures therefore suggest that glucose (1 molecule) gives rise to 1 molecule each of lactic acid, acetaldehyde and formic acid, the aldehyde being converted to ethyl alcohol and acetic acid and the formic acid to $\text{CO}_2 + \text{H}_2$.

Cook and Stephenson showed that formic acid in presence of *Bact. coli* was completely but lactic and succinic acids only partially oxidized to CO_2 . Stickland found that H_2 was produced by *Bact. coli* from formates only in presence of the proliferating cell and that under strictly aerobic conditions the organism gave no free H_2 . Hence although "resting" *Bact. coli* had a powerful activating influence on formic acid, the breakdown to H_2 did not take place. *Bact. typhosum*, even when proliferating, cannot form free H_2 from HCOOH although having a powerful activating action on the acid. Some factor present in *Bact. coli* but absent from *Bact. typhosum* is therefore partly responsible for the liberation of free H_2 . Stephenson and Stickland showed that this factor was probably an enzyme (hydrogenase) and the liberation of H_2 then required the combined action of formic acid

dehydrogenase and hydrogenase. This view does not explain, however, why free H_2 is formed only in presence of the proliferating cell.

(d) **Production of Fatty Acids.**—1. *Acetic Acid.*—The acidification of dilute alcohol solutions on standing in air was known a long time before Kützing and later Thomsen showed that the phenomenon Pasteur's "mycoderma aceti" *Acetobacter aceti* and *Acetobacter pasteurianum*, while Brown worked with *Acetobacter xylinum*, Hansen with *Acetobacter Kützingianum* and at later dates numbers of organisms capable of oxidizing ethyl alcohol to acetic acid have been isolated. The production of ethyl alcohol and acetic acid by way of acetaldehyde (Harden) by many types of bacteria has already been mentioned, while most of the fermentations producing $H_2 + CH_4$ also yield this acid. Thus all those mesophilic anaerobes, e. g., *Clostridium pasteurianum*, *Plectridium pectinovorum* and *Granulobacter pectinovorum* which have been grouped by Bredemann in one species as *B. amylobacter* can ferment pectins, starches, hexoses, pentoses, etc., yielding besides butyl alcohol and acetone considerable amounts of acetic acid. Khouvine also showed that *B. cellulosa dissolvens* ferments 1.012 Gm. cellulose to give 0.275 Gm. acetic acid and 0.033 Gm. butyric acid. The formation of acetic acid in thermophilic fermentations, during the decomposition of vegetable wastes, in ensilage, by fermentation of cotton cobs, during retting, etc., is dealt with in Thaysen and Bunker's book.¹

The more recent work has dealt with the mechanism of the conversion of ethyl alcohol to acetic acid. The first reaction, a dehydrogenation, results in the conversion of ethyl alcohol to acetaldehyde. Neuberg and Nord by their sulfite method succeeded in demonstrating the intermediate formation of aldehyde. Acetaldehyde may then either undergo dismutation, $2CH_3CHO + H_2O = CH_3.COOH + CH_3CH_2OH$, or its hydrate may undergo a second dehydrogenation, $CH_3CH(OH)_2 \rightarrow CH_3COOH$, thus giving rise to acetic acid. From a study of the relative velocities of dehydrogenation and dismutation reactions, it can be shown that dismutation plays only a very unimportant part in the acetic acid fermentation by *Acetobacter orleanse*, *pasteurianum* and *ascendens*.

2. *Propionic Acid.*—Bacteria of various groups occurring especially in milk, cheese, rennet, etc., can ferment various substrates; e. g., carbohydrates, lactic, tartaric and quinic acids, glycerol, proteins and their degradation products to give propionic, acetic and carbonic acids. Hydrogen, however, is never formed. A detailed description of these reactions is given in Van Niel's monograph,⁷ and a useful summary in Kluyver's collected lectures.⁹ Fitz attempted to investigate the propionic acid fermentation quantitatively and, using lactic acid as substrate, suggested an equation according to which 3 molecules of lactic acid give rise to 2 molecules propionic acid with 1 molecule each of acetic acid, CO_2 and H_2O . Freudenreich and Orla Jensen, working with Emmental cheese, were the first to effect the rather difficult iso-

lation of pure cultures of the organisms responsible for the presence of large amounts of volatile acid. Assuming Fitz's equation for the fermentation of lactic acid is correct, propionic acid must arise by direct hydrogenation of lactic acid $\text{CH}_3\text{CHOHCOOH} + 2\text{H} = \text{CH}_3\text{CH}_2\text{COOH} + \text{H}_2\text{O}$; other molecules of lactic acid acting as hydrogen donators and by so doing being converted into acetic acid and CO_2 . $\text{CH}_3\text{C}(\text{OH})_2\text{COOH} \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 + 2\text{H}$.

Careful analysis, however, shows that the ratio of propionic to acetic acid does not exactly correspond to the ratio of 2:1 as is required by Fitz's equation, but is generally about 1.8. The function of lactic acid as a hydrogen acceptor must, therefore, be partly taken over by some other hydrogen acceptor present in the medium. It was found that aspartic acid or asparagine in the yeast extract acted in this way and gave rise to succinic acid. Passing next to the fermentation of glucose, it is found that although the same products are formed, still the ratio of propionic to acetic acid often considerably exceeds Fitz's ratio of 2, with different strains varying from 1.9 to 4.2. It seems therefore impossible to explain the glucose fermentation by considering it as a fermentation of primarily formed lactic acid, and it seems certain that nutrient substances must act as hydrogen donators. After careful investigation, Van Niel concluded that readily reducible compounds, *e. g.*, glyceraldehyde, methylglyoxalhydrate, are first formed and become hydrogenated to propionic acid, other organic compounds in the yeast extract being dehydrogenated. This view is supported by the fact that when yeast substances of a previous experiment are used for a second experiment, whereas the ratio of propionic to acetic acid was 3.8 in the first experiment, it fell to the theoretical value of 2 in the second fermentation.

A further interesting fermentation by these bacteria is that of glycerol, carried out by Van Niel, in which the glycerol fermented is almost quantitatively converted to propionic acid. It is concluded that 1 molecule of glycerol is dehydrogenated to form glyceric aldehyde which isomerizes to methylglyoxalhydrate which is then hydrogenated by 2H from a second molecule of glycerol, thus giving propionic acid.

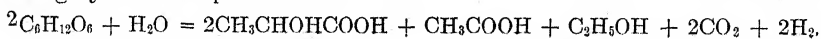
Investigation of the fermentation of pure sugars and of mixtures containing sugar (*e. g.*, molasses, hydrolyzed starch, etc.) by 11 strains of propionic acid bacteria shows that glucose, lactose, maltose and sucrose are only partially utilized by pure cultures but almost completely fermented by mixtures of these cultures with *Lactobacillus casei*, up to 75 per cent of the sugar consumed being converted into volatile acids consisting of acetic and propionic acids in the molecular proportion of 1:2.46.

3. Butyric Acid.—The fermentation of glucose by butyric acid bacteria in the more restricted sense of the name leads to the formation of only four chief products, namely butyric and acetic acids, CO_2 and hydrogen, while traces of formic acid are detected and the production of alcohol is almost negligible. The other species of the group produce

in the butyl alcohol-acetone fermentation as many as 10 different substances. The production of butyric acid from glucose was first noticed by Pasteur and formed the earliest example of a strictly anaerobic fermentation. It was Fitz, however, who first investigated the chemistry of the fermentation. He isolated an organism which from glycerol, mannitol, starch and invert sugar produced *n*-butyl alcohol, *n*-butyric acid and traces of ethyl alcohol and possibly of caproic acid. With another organism he obtained butyric and propionic acids from lactate and with *B. subtilis* obtained butyric acid from starch.

Buchner and Meisenheimer found that from glycerol more butyl and ethyl alcohols were produced by Fitz's organism while from glucose more butyric and acetic acids were formed. The production of 4-carbon compounds from glycerol and lactic acid led necessarily to the belief that butyric acid arises by some condensation process probably analogous to the aldol condensation. Neuberg and Arinstein, using the sulfite fixation method with fermentations by *B. butylicus* Fitz showed that acetaldehyde was an intermediate product and that in the presence of sulfite, aldehyde was fixed and no 4-carbon compounds formed. They found also that addition of pyruvic acid during the fermentation only led to the formation of acetic and formic acids whereas addition of pyruvic aldol gave a good yield of butyric acid. There seems little doubt, therefore, that 4-carbon compounds do arise by condensation of molecules either of acetaldehyde or of pyruvic acid, or of mixtures of these, the formation of higher fatty acids being thus brought into line with their formation in the animal body and in the green plant. The mechanism of the reactions involved has been studied by Donker, and a summary is given in Kluyver's collected lectures.⁹ The scheme of reactions proposed by these authors is as follows: Glucose by an internal mechanism first gives rise to 2 molecules of methylglyoxalhydrate which is then decomposed according to the equation $\text{CH}_3\text{COCH}(\text{OH})_2 \rightarrow \text{CH}_3\text{CHO} + \text{HCOOH}$. Formic acid gives rise to $\text{CO}_2 + \text{H}_2$ while acetaldehyde on the one hand hydrates and gives acetic acid and hydrogen and on the other condenses with itself to give acetaldol which isomerizes to butyric acid. All these reactions are of the hydrogen transference type and much quantitative evidence is adduced favoring the proportion of products required by the scheme.

4. *Lactic Acid*.—Organisms producing lactic acid from glucose may be roughly divided into two classes, those, *e. g.*, *Bact. acidi lactici*, *Bact. coli*, *Bact. lactis aerogenes*, *Bact. cloacae*, *Lactobacillus pentaceticus*, etc., which give amounts of acid corresponding to only a half (or less) of the sugar fermented and true "lactic acid fermenters," *e. g.*, the two anaerobic organisms described by Kayser which decompose hexoses almost quantitatively to lactic acid. Working with *Bact. coli communis*, Harden showed that the fermentation reaction corresponded roughly to the equation:



Harden's figures suggest that 1 molecule of glucose gives rise to 1 molecule each of lactic acid, acetaldehyde and formic acid, the aldehyde being the precursor of alcohol and acetic acid, the formic acid of CO_2 and hydrogen. Neuberg and Gorr, using a heavy suspension of *Bact. coli*, effected a quantitative conversion of methylglyoxal to lactic acid, and the glyoxal may therefore be the precursor of lactic acid. Using *Bact. coli* and mannitol, Grey obtained similar results but less lactic acid and more alcohol; while using glucose, gluconic acid, glycuronic acid and saccharic acid, Kay showed that as we pass from the more reduced to the more oxidized substrate, the amounts of lactic acid formed decrease and those of succinic and acetic acids increase.

Carbon balance sheets showing the amount of lactic acid produced from glucose by a variety of organisms are given by Birkinshaw, Charles and Clutterbuck.¹³ Another group of organisms, the pentose fermenters, *Lactobacillus pentoaceticus*, which have been studied by Peterson and Fred,¹⁷ break down aldohexoses in a manner very like that of *Bact. coli*, the equation for the reaction being with glucose $\text{C}_6\text{H}_{12}\text{O}_6 = \text{CH}_3\text{CHOHCOOH} + \text{C}_2\text{H}_5\text{OH} + \text{CO}_2$. Substitution of fructose for glucose leads to the replacement of alcohol by acetic acid, and reduction of part of the fructose to mannitol. Sucrose does not give mannitol unless first inverted so that sucrose must be attacked directly by bacteria. The scheme of reactions with fructose is:

1. $\text{C}_6\text{H}_{12}\text{O}_6 + \text{H}_2\text{O} = \text{CH}_3\text{COOH} + \text{COOHCH}_2\text{CHOHCOOH} + 2\text{H}_2$.
2. $\text{COOHCH}_2\text{CHOHCOOH} = \text{CH}_3\text{CHOHCOOH} + \text{CO}_2$.
3. $2\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2 = 2\text{C}_6\text{H}_{14}\text{O}_6$.

The manufacture of lactic acid has been described* by Garrett. The process, which is essentially an American one, consists of the high temperature fermentation of cane or beet sugar, molasses or of corn starch (after hydrolysis with acid or diastase) by pure cultures usually of *Lactobacillus delbrueckii*, this organism being chosen because of its vigorous fermenting activity and of its high optimum temperature which retards the growth of other contaminating bacteria and results in a relatively pure grade of lactic acid. In Germany potato starch is often used and the use of dairy industry wheys has been tried, but here the sugar content is rather low and an organism of the *Bulgaricus* type must be used since *L. delbrueckii* does not ferment lactose. In addition to sugar, nitrogen and salts are added, usually as gluten from grains or ammonia and phosphates. The acid is neutralized as formed either by addition of calcium carbonate or by the gradual addition of lime. The fermentation requires five to six days. Eighty to 90 per cent of the entire consumption of the acid is used in the removal of lime from dehaired hides. The acid is also used in chrome mordanting and in acid dyeing of wools, in the production of ethyl lactate as a solvent for nitrocellulose in pyroxylin lacquers, and in various food and beverage products.

(e) **Butyl Alcohol-acetone Fermentation.**—Butyl alcohol was first shown to be present in fusel oil by Wurz in 1852 and was obtained as a normal product of the butyric fermentation of lactic acid by Pasteur in 1862 and of glycerol by Fitz in a series of papers from 1876–1884. His organisms did not, however, attack starch. Schardinger was the first to isolate a bacillus, *B. macerans*, able to ferment starch giving rise to acetone. At a later date, both these substances assumed commercial importance. Butyl alcohol was required for formation of butadiene in the synthesis of artificial rubber, and subsequently as butyl acetate and phthalate became valuable solvents in the production of lacquers and varnishes, while acetone was needed during the war for aeroplane dope and for smokeless powders.

The three organisms of Fernbach and Strange, Weizmann and of Reilly, Higginbottom, Henley and Thaysen¹⁵ may be grouped together and convert hexose (hydrolyzed starch) into ethyl and butyl alcohols, acetone, acetic and formic acids, CO₂ and hydrogen. Reilly found that addition of CaCO₃ to the mash caused a decrease of acetone and butyl alcohol production and an increase of volatile acids while addition of acetic acid led to a considerable increase of acetone production. Using another organism, *Granulobacter pectinovorum*, Speakman¹⁶ obtained results very similar to those of Reilly. Northrop, Ashe and Senior isolated another bacillus, *B. acetoehtylicum*, which hydrolyzes starch and ferments the hexoses formed giving acetone and ethyl alcohol but no butyric acid or butyl alcohol. Using the same organism, Arzberger, Peterson and Fred demonstrated the effect of pH on the amounts of volatile acid, alcohol and acetone produced. At a later date Wynne investigated the effect of addition of numerous organic and inorganic acids on the fermentation, and Speakman showed that in the fermentation of sugar, the formation of pyruvic acid preceded that of acetone. The same author showed that whereas in the fermentation of glycerol only traces of acetone appeared, yet on addition to this fermentation of pyruvate, acetone was formed in considerable amounts. He suggested, therefore, that acetone arises from pyruvic acid by way of acetaldehyde, aldol, β -hydroxybutyric and acetoacetic acids. Bakonyi, however, criticized this view on the ground that β -hydroxybutyric acid is not readily attacked by this organism; and he suggests rather that the aldol is dismutated to ethyl alcohol and acetic acid, the latter then giving rise to acetone.

It seems certain from the work of the various authors that acetone arises from acetic acid and that butyl, ethyl and isopropyl alcohols are formed by hydrogenation of butyric acid, acetaldehyde and acetone respectively. From a careful quantitative study of the reactions Kluyver, Donker and Van der Lek have suggested the following scheme of reactions, a scheme which fits the quantitative evidence and rules out many of the alternative schemes. Glucose first gives rise by internal rearrangement to 2 molecules of methylglyoxalhydrate, $C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$, which then passes to acetaldehyde and formic acid,

$\text{CH}_3\text{COCH}(\text{OH})_2 \rightarrow \text{CH}_3\text{CHO} + \text{HCOOH}$. The subsequent reactions may be divided into:

(A) *Dehydrogenation* reactions including $\text{HCOOH} \rightarrow \text{CO}_2 + 2\text{H}$:
 $\text{CH}_3\text{CH}(\text{OH})_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}$.

(B) *Condensation* reactions $2\text{CH}_3\text{CHO} \rightarrow \text{CH}_3\text{CHOHCH}_2\text{CHO} \rightarrow \text{CH}_3\text{CH} = \text{CHCH}(\text{OH})_2 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH}$; $2\text{CH}_3\text{COOH} \rightarrow \text{CH}_3\text{C}(\text{OH})_2\text{CH}_2\text{COOH} \rightarrow \text{CH}_3\text{COCH}_2\text{COOH} \rightarrow \text{CH}_3\text{COCH}_3 + \text{CO}_2$ and

(C) *Hydrogenation* reactions $2\text{H} \rightarrow \text{H}_2$; $\text{CH}_3\text{CHO} + 2\text{H} \rightarrow \text{C}_2\text{H}_5\text{OH}$; $\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 4\text{H} \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$ and $\text{CH}_3\text{COCH}_3 + 2\text{H} \rightarrow \text{CH}_3\text{CHOHCH}_3$.

The *industrial fermentation* is particularly interesting as a process in which maize is converted almost entirely into useful products. Thus the chief products butyl alcohol, acetone and ethyl alcohol formed in the proportion of 6:3:1 make up 25-30 per cent of the starch. All the hydrogen together with part of the CO_2 are converted into methanol and the remainder of the CO_2 into dry ice. The maize germ is separated and the oil expressed and used for food purposes, the residual oil-cake meal for cattle fodder. These products, together with the distillery slop, amount to 96 per cent of the dry weight of the corn.

(f) **Production of 1-Acetylmethylcarbinol, 2:3-Butyleneglycol and Dihydroxyacetone.**—1. *1-Acetylmethylcarbinol and 2:3-Butyleneglycol.*—Grimbert, growing *B. tartricus* on solutions containing peptone and carbohydrate, isolated the carbinol as its osazone and Desmottz obtained it similarly using *B. subtilis* and *Tyrothrix tenuis*. Harden and Walpole, in *Bact. lactis aerogenes* fermentations of glucose, obtained one third of the glucose carbon as a mixture of carbinol and glycol, and Walpole separated the glycol into two optically inactive glycols. Harden and Harden and Norris showed that the carbinol is responsible for the Voges-Proskauer reaction given by many bacteria, the carbinol being oxidized in alkaline solution to diacetyl which combines with constituents of the broth containing the guanidine nucleus. The mechanism of the test has recently been questioned. Harden and Norris also found that the glycol was formed by *Bact. lactis aerogenes* in a peptone chalk medium, when acetaldehyde was gradually added and Neuberg and Hirsch obtained with yeast a condensation of acetaldehyde and benzaldehyde to *l*-phenylacetylcarbinol, the enzyme responsible being named carboligase. The amount of the carbinol normally produced by yeast and determined by conversion to diacetyl and precipitation as the nickel glyoxime can be considerably increased by addition to fermenting yeast of pyruvic acid or of acetaldehyde. Walpole showed that aeration favored the formation of carbinol by *Bact. lactis aerogenes* and Kluyver, Donker, etc., found that its production by yeast was increased by addition of hydrogen acceptors such as methylene blue. Elion showed that well-aerated yeast suspensions oxidize aqueous solutions of alcohol to acetaldehyde and condensation to the carbinol occurs, the simultaneous fermentation of

sugar being unnecessary. Finally, the production of butyleneglycol by a number of bacteria is quantitatively demonstrated in the carbon balance sheet experiments of Birkinshaw, Charles and Clutterbuck.¹³ It was found that *B. asiaticus mobilis* gave about a 30 per cent yield of glycol and the rate of fermentation was much greater than for any other organism examined.

2. *Dihydroxyacetone*.—A short summary of work on the bacterial oxidation of glycerol to dihydroxyacetone is given in Bernhauer's monograph.⁸ The reaction was first noted by Bertrand using the sorbose bacterium (identical with *Acetobacter xylinum*) and a careful description of its preparation was given later. Similar reactions are recorded by Watermann using *Acetobacter melanogenum* Beyerinck, and by Fernbach using *Tyrophrix tenuis*. Agulhon and Sazerac showed that the reaction with the sorbose bacterium was accelerated by traces of uranium salts ($\frac{1}{1000}$). After working out the optimum conditions for the reaction, Virtanen and Bärlund using *B. dioxyaceticum* obtained an 85 per cent, Bernhauer and Schön using *A. xylinum* a 100 per cent, and Hermann and Neuschal using *A. gluconicum*, *xylinum*, *xylinoides*, *orleanse* and *aceti* a 90 per cent yield of dihydroxyacetone.

(g) **Production of Mannitol from Fructose**.—The production of mannitol was early noted by Pasteur as a product of the so-called "viscous fermentation" in which 100 parts of sucrose give 51 parts of mannitol, the remainder being carbohydrate gum. Gayon and Dubourg and later Laborde showed that the "maladies des vins" were due to the presence of bacteria which were able to attack glucose and fructose giving respectively glycerol and mannitol and not ethyl alcohol. These organisms acting on fructose gave 60–70 per cent yield of mannitol; and although giving mannitol with invert sugar, gave no mannitol with sucrose, this possibly being attacked without preliminary inversion. Working with a group of pentose fermenters, *Lactobacillus pentoaceticus*, Peterson, Fred and their colleagues¹⁸ found that although glucose was fermented in the same way as with *Bact. coli*, fructose which can itself act as a hydrogen acceptor, was partly reduced to mannitol.

(h) **Gluconic Acid**.—Boutroux, using *Mycoderma aceti* and *Micrococcus oblongus*, was the first to isolate gluconic acid from glucose fermentations; and it has since been frequently obtained. The bacterial production of gluconic acid forms another of the oxidational fermentations dealt with in Bernhauer's monograph.⁸

(i) **Bacterial Synthesis of Carbohydrates, etc.**—The bacterial slimes or gums which consist chiefly of polysaccharides were early studied by Pasteur and Van Tieghem. Ruhland, growing *B. spongiosus* on broth agar with 20 per cent sucrose, isolated an araban. Schardinger with a member of the lactis aerogenes group obtained a galactan. Fernbach and Schoen, using *Gommobacter*, obtained from sucrose but not from glucose, fructose or invert sugar a laevulan; and Fernbach, Schoen and Hagiwari with *Leuconostoc dextranicus* a dextran. Grieg Smith iso-

lated an organism from the gum acacia tree which on a synthetic medium gave a gum identical with true gum acacia, and giving on hydrolysis arabinose and galactose. Buchanan also obtained a nitrogen-free gum by growth of *B. radicola* on a synthetic medium containing dextrose, fructose, galactose, maltose, sucrose, lactose, mannitol, glycerol, amygdalin, salicin, citrate or succinate. Mucilages containing nitrogen were early reported using *Ps. fluorescens* and *B. glyscrogenum*. It is impossible to review here in any detail which will do justice to the subject, the numerous studies which have followed the isolation, by Avery, Heidelberger and their colleagues¹⁹ of type specific polysaccharides from pneumococci, and by various other workers of similar components from a variety of bacterial species. The most recent development of this work has been the discovery by Avery and Goebel²¹ of the acetylated form of the type I pneumococcal polysaccharide and by Raistrick and Topley²² and Boivin and his colleagues, of a complex carbohydrate free from protein which has been isolated from *Bact. aertrycke*. Both of these polysaccharides have very marked immunizing properties.

II. ACTION OF BACTERIA ON NITROGENOUS SUBSTRATES

General Amino-acid Breakdown by Bacteria.—Work in this direction has been ably summarized by Stephenson⁵ and Buchanan and Fulmer.² The degradation of protein by micro-organisms involves first the cleavage to amino acids, carried out by proteolytic enzymes acting outside the cell, and second the reduction, oxidation, decarboxylation, deamination, etc., of these acids by mechanisms which appear to involve cell structure. The chief types of bacterial decomposition of amino acids may be divided into I, decarboxylation giving an amine; II, hydrolytic deamination giving the hydroxy-acid; III, hydrolytic deamination and decarboxylation giving the alcohol; IV, reductive deamination giving the saturated acid; V, reductive deamination and decarboxylation giving a hydrocarbon; VI, deamination at the $\alpha\beta$ -linkages giving the unsaturated acid; and VII, oxidative breakdown giving compounds having fewer carbon atoms.

Using glycine as substrate, reactions of type I are recorded with *B. fluorescens liquifaciens* on gelatin, with *Streptococcus longus* on fibrin and with mixed cultures in the putrefaction of fish, while reactions of type IV are given by *Cl. putrificum* and of type V under anaerobic conditions using *Rauschbrand's bacillus* on elastin and *Clostridium carnofoetidum* on fibrin. Using alanine, reactions of type I are given with mixed cultures in the putrefaction of wheat flour, of type IV by *Cl. putrificum* and of type VII with *B. proteus*. Using valine, reactions of type I and type IV are given with mixed cultures and of type VII with *Proteus vulgaris*. With leucine, reactions of type I are recorded with mixed cultures in the putrefaction of meat and with *Proteus vulgaris* and *B. subtilis*, of type II with *Proteus vulgaris* and *Granulobacter pectinovorum* and of type III chiefly with yeasts. With

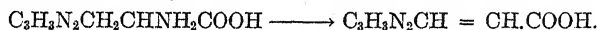
isoleucine, reactions of type IV and of type VII are given with mixed cultures. Using serine, reactions of types IV and VII are given with *Cl. putrificum*. Using aspartic acid, reactions of type I are given with mixed cultures of type II with *Bact. coli*, of type IV with *Proteus vulgaris*, *Cl. putrificum*, *B. fluorescens*, with various mixed cultures, and with certain facultative and strict anaerobes, of type V with mixed cultures and with *Cl. putrificum*, of type VI with *Bact. coli* and of type VII with *Proteus vulgaris* and *Cl. putrificum*.

• Using glutamic acid, reactions of type I and of type IV are given with mixed cultures, of type V with mixed cultures, and with *Cl. putrificum* and of type VII with mixed cultures. Using ornithine, reactions of type I and of type IV are given with mixed cultures. With lysine, reactions of type I and of type IV are given by mixed cultures. Using histidine, reactions of type I are given with mixed cultures, with *B. aminophilus intestinalis*, with an organism of the typhosus group and with a strain of *Bact. coli*, of type II with *Proteus vulgaris*, of type III by yeast, of type IV with mixed cultures and a strain of *Bact. coli*, of type VI with *Bact. coli*, *Bact. typhosum*, *Bact. paratyphosum* (A and B), *Bact. enteritidis* and *Bact. dysenteriae*,¹⁰ and of type VII with *Proteus vulgaris*, organisms of the *coli*-typhosus group, *Alkaligenes faecalis* and *Ps. pyocyanea*.¹⁰ Using phenylalanine, reactions of type I are given with mixed cultures in the putrefaction of gelatin, meat, and placenta, of type II with *B. subtilis* and *Proteus vulgaris*, of type III with yeast, of type IV and type VII with mixed cultures during putrefaction of meat and keratin. Using tryptophan, reactions of type I are given with *B. aminophilus intestinalis*, of type II with *Proteus vulgaris*, of type III with yeast, of type IV with *Bact. coli* and of type VII with mixed cultures in the putrefaction of proteins and of ox brain, with *Bact. coli*, *Ps. pyocyanea* and *Ps. fluorescens* and with *B. subtilis*. Using tyrosine, reactions of type I are given by mixed cultures in the putrefaction of meat and placenta, by *B. aminophilus intestinalis*, by *Bact. coli* and *Proteus vulgaris* and by a cheese bacillus, of type III by *Proteus vulgaris* and by *B. subtilis*, of type III by yeast, of type IV by *Cl. putrificum* and a bacillus resembling *Ps. pyocyanea*, of type VI by *Proteus vulgaris* and of type VII by a mixed culture in keratin putrefaction, by *Proteus vulgaris*, by a bacillus resembling *Ps. pyocyanea*, by *B. phenologenes* and by *B. coli phenologenes*.

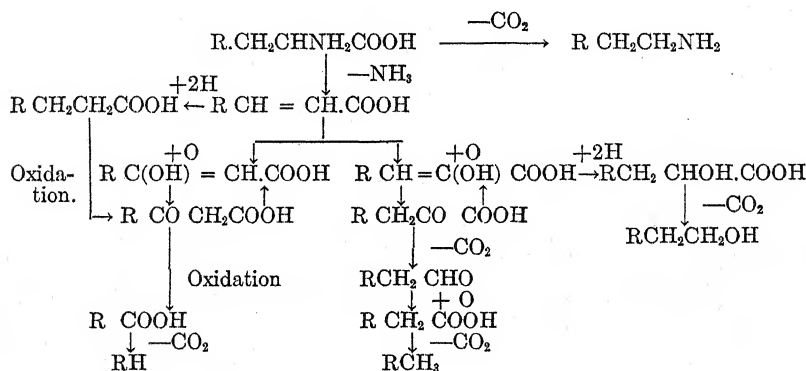
The conversion of arginine to ornithine has been effected by mixed cultures, of proline to γ -aminovalerianic and valerianic acids by mixed cultures, and the production of hydrogen sulfide and possibly methylmercaptan from cysteine has been described. A study of these reactions shows that whereas molds frequently hydrolytically deaminate amino acids giving *d*-hydroxy-acids and yeasts commonly hydrolytically deaminate and decarboxylate giving alcohols, bacteria often effect hydrolytic deamination but never hydrolytic deamination together with decarboxylation. Using both strict and facultative anaerobes, under anaerobic conditions deamination is accompanied by

reduction giving the saturated acid. Under aerobic conditions, the course of the reaction is largely influenced by the other constituents of the medium and saturated acids form only one of several degradation products.

Although no complete general scheme of bacterial breakdown of amino acids can be given with certainty, Raistrick's work has suggested the following.¹⁰ Using heavy inoculations of organisms of the coli-typhosus group in a Ringer's solution-histidine medium, considerable amounts of the corresponding unsaturated (urocanic) acid were obtained. The primary cleavage appears therefore to be a loss of ammonia with desaturation at the α - β -linkage:



A similar reaction occurs in the conversion of tyrosine into *p*-hydroxyphenylacrylic acid and of aspartic to fumaric acid. The remainder of the products may then arise as follows:



Considerable evidence for aerobic decomposition to α -keto-acids and the conversion by bacteria of these acids into formic acid and a fatty acid having one carbon less than the keto-acid has already been dealt with.

Perhaps one of the most interesting degradations is that of tryptophan. The formation of indole and skatole was early recognized by Kühne and the production of indole from tryptophan has long been used as a diagnostic test for certain species of bacteria. The formation on meat broth with mixed putrefaction cultures of indoleacetic acid was also recorded by Salkowski. In 1903 Hopkins and Cole¹² isolated tryptophan from tryptic digests of casein and showed that *Bact. coli* under aerobic conditions formed from tryptophan, indole and indoleacetic acid and under anaerobic conditions indolepropionic acid. The breakdown of the indole nucleus has been investigated by Raistrick and Clark in their quantitative experiments using *Ps. pyocyanea*, *Ps.*

fluorescens, *Chromobacterium prodigiosum* and *Proteus vulgaris*. The first two organisms attacked both the side chain and the nucleus while *Proteus vulgaris* attacked only the side chain.

METABOLISM OF YEASTS

I. ACTION OF YEASTS ON NONNITROGENOUS SUBSTRATES

(a) **Alcoholic Fermentation.**—1. *General (Rôle of Phosphates, Coenzyme, etc.)*.—Since Buchner first demonstrated that alcoholic fermentation could be carried on in the absence of yeast cells, an enormous amount of research has been directed to the study of the mechanism of the reactions involved and a comprehensive summary is given by Harden²³ while some of the more recent work is discussed by Meyerhof.²⁸

In their early experiments, Harden and Young found that yeast juice could be readily divided by ultrafiltration into a residue containing protein, glycogen, dextrans, enzymes (proteases, hexosephosphatases, etc.) but no mineral phosphate, and a filtrate containing besides phosphate a dialyzable thermostable substance termed the coenzyme (cozymase). Neither fraction alone effected fermentation but on mixing, activity reappeared. Euler and his colleagues²⁴ and also Meyerhof and Iwasaki and Abderhalden found that yeast also contained other substances—Z factors—which accelerate the fermentation of living but not of dried yeast whereas coenzyme has no such action.

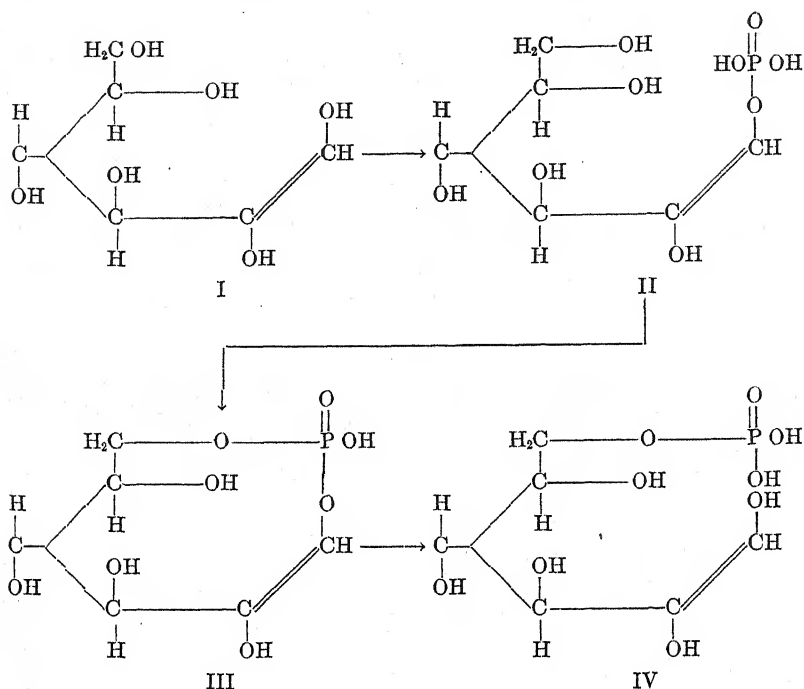
A considerable amount of work by Euler and Myrbäck²⁴ has led to the purification of the coenzyme which consists chiefly of a substance of the nature of an adenylnucleotide (adenylic acid). According to Lohmann, adenylic acid (or adenylic pyrophosphate) requires (as complement) the presence of magnesium and Euler and Nilsson, while agreeing to the necessity for magnesium, maintain that for full activation a substance related to Euler's "Z factors" is also necessary.

Buchner's yeast juice, Lebedew's maceration extract, Albert's zymine and dried yeast all ferment sugar more slowly than living yeast and all respond to the presence of inorganic phosphate giving rise to phosphoric esters of the sugar. Thus Harden and Macfarlane showed that expressed juice ferments sugar at one twentieth to one fortieth of the rate of living yeast but in presence of phosphate this low rate may be increased to half the rate of living yeast. Treatment with toluene also gradually kills the yeast cells, the rate of fermentation falls and the response to phosphate is acquired. The high rate of fermentation occurs therefore in presence of a suitably high concentration of phosphate. When this is depleted by conversion into sugar phosphoric ester, the rate falls to the low level corresponding to the slow regeneration of phosphate from the phosphoric esters and only increases again either on addition of more phosphate, or for example by addition of arsenate which accelerates the regeneration of phosphates from the esters.

The amount of sugar fermented is approximately equivalent to the amount of phosphoric acid esterified in the ratio of $C_6H_{12}O_6/2PO_4$, or since one molecule sugar gives 2 molecules CO_2 , the CO_2 evolved is equivalent to the phosphoric acid esterified, $CO_2/PO_4 = 1$. Harden and Henley support this view but Kluyver and Struyk report ratios as low as 0.6. To obtain really accurate figures is, however, difficult since there is no satisfactory way of completely separating the primary rapid fermentation with esterification from the secondary slow fermentation of the hexosephosphate formed. Boyland observed that a preparation can sometimes be obtained by acetone precipitation of yeast juice which is almost devoid of the power of regenerating phosphate and further work on these lines is desirable. The ratio is, however, remarkable in that the products of different experiments for which the ratio holds are often of different composition.

Various hexosephosphates have been described. Using yeast juice, Harden and Young obtained hexosediphosphate, probably the 1:6-diphosphoric ester of fructofuranose which, however, does not appear to exist in either living yeast or in other intact cells or tissues. More recently Harden and Robison isolated also a hexosemonophosphate shown by Robison to consist of a mixture of roughly 70 per cent of an aldose ester (glucose-6-phosphate) and a ketose ester together with small amounts of two other substances. This mixture of hexosephosphates is practically identical with Embden's "lactacidogen" obtained from muscle. Still later, Robison²⁵ obtained a mannosephosphate, probably possessing the phosphate group in position 6; and also, as a component of the fermentation hexosemonophosphate, the same fructosephosphate (6-phosphoric ester of fructofuranose) as was previously obtained by Neuberg by partial hydrolysis of hexosediphosphate. Finally, using dried yeast and glucose or fructose with phosphate, Robison and Morgan showed that 80-90 per cent of the phosphoric esters consisted of hexosediphosphoric acid and that the remainder consisted partly of Robison's monophosphate along with trehalosemonophosphoric ester. This latter ester with bone phosphatase gives trehalose and on acid hydrolysis gives glucose and a glucosemonophosphate identical with the aldose constituent of the Robison ester. Glucose-3-phosphoric acid has also been prepared by synthetic means and from sucrosemonophosphoric acid.

A scheme for the formation of the four esters in fermentations by yeast juice has been suggested by Robison.²⁵ It is based on Armstrong's suggestion that the substance actually fermented by yeast is the enolic compound common to glucose, fructose and mannose (I). It is supposed that 1 molecule of phosphoric acid is attached as in II, that this then wanders via III to the 6-position. The resulting ester (IV) by addition of water would yield the hydrated form of glucose-, mannose- and fructosephosphates, and removal of water 6-phosphoglycopyranose, 6-phosphomannopyranose and 6-phosphofructofuranose:



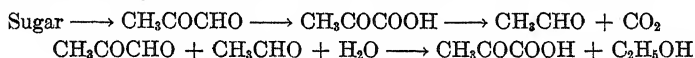
Moreover, the enolic group of IV could react with a further molecule of phosphoric acid giving the diphosphoric ester which would yield 1: 6-diphosphofructofuranose, the hexosediphosphate of Harden and Young. Hydrolysis of these esters by yeast or bone phosphatases reverses these reactions.

The interrelationship of the esters has been studied in detail by Lohmann. Thus the same equilibrium mixture of ketose and aldose esters is formed in a few seconds from either the pure glucose or fructose ester in the presence of an enzyme found in all cells. Enzymic hydrolysis of hexosediphosphate also gives the same equilibrium mixture. Dialyzed muscle extract has no effect on hexosediphosphate in the absence of magnesium and adenylypyrophosphate, but on adding magnesium, one phosphoric acid group is split off. The Neuberg ester, on the other hand, is converted into the Robison ester in a few seconds even in absence of magnesium.

Esterification with phosphoric acid to give these products is regarded, therefore, as the first step in the degradation of carbohydrates both in alcoholic fermentation and in the formation of lactic acid in muscle, and we now pass to the intermediary steps in the breakdown. These have been worked out largely with muscle extracts and the results are now being successfully applied, with certain differences, to yeast fermentation.

The discovery of methylglyoxalase by Dakin and Dudley and inde-

pendently by Neuberg led to the view that the formation of lactic acid in muscle, and of pyruvic acid and acetaldehyde in alcoholic fermentation probably occurred by way of methylglyoxal. Working with magnesium hexosediphosphate and extracts of yeast plasmolyzed by ether, dried yeast, maceration extract, etc., Neuberg and Kobel²⁶ isolated good yields of methylglyoxal as the bisdinitrophenylhydrazone. A similar production of the glyoxal using lactic acid-forming bacteria²⁶ and during glycolysis by animal tissues has also been demonstrated. A vast amount of work has been done by Neuberg and his collaborators in this connection, and the view held until recently was that sugar was first degraded to methylglyoxal, which was converted by oxidation-reduction into pyruvic acid, which in presence of carboxylase gave acetaldehyde and CO_2 . The aldehyde was then reduced to alcohol by the same oxidation-reduction reaction in which the glyoxal was oxidized to pyruvic acid, and the reaction thus became continuous:

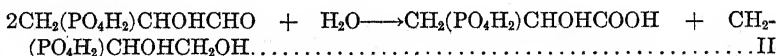


The most recent work, however, which is due to Embden²⁷ and to Meyerhof,²⁹ suggests that methylglyoxal is not the intermediate substance formed. Thus muscle extracts from which glutathione has been removed can readily convert glycogen but cannot convert methylglyoxal to lactic acid. For the latter reaction glutathione which, as Lohmann has shown, acts as a true coenzyme of methylglyoxalase, must be added. A summary of these views has been given by Meyerhof.²⁸

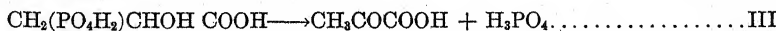
The new scheme of fermentation may be briefly summarized as follows. In the reaction of sugar with phosphate, phosphorylation first occurs and then breakdown, probably to phosphoglyceraldehyde.



In the early stages of fermentation before any acetaldehyde has been formed, 2 molecules of phosphoglyceraldehyde undergo an oxido-reduction giving rise to a molecule each of phosphoglyceric acid and phosphoglycerol.



Phosphoglyceric acid is next attacked by the yeast enzymes giving rise to pyruvic and phosphoric acids.

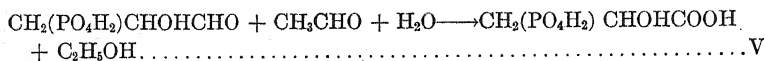


In presence of carboxylase, pyruvic acid yields aldehyde and CO_2 .



As soon as sufficient acetaldehyde has been produced in this way reaction II stops and only small amounts of phosphoglycerol are ever produced, and correspond probably with the small amounts of glycerol found in all alcoholic fermentations. In place of reaction II the oxido-

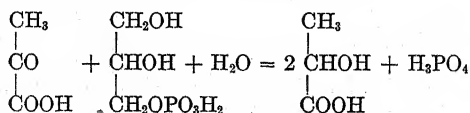
reduction reaction occurs between 1 molecule each of phosphoglycer-aldehyde and acetaldehyde, with the production of phosphoglyceric acid and alcohol.



The phosphoglyceric acid thus reacts as in III and so the cycle is established.

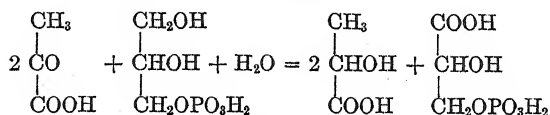
We will now review the evidence on which the above scheme is based. Embden observed a long time ago that sodium fluoride inhibited lactic acid production by muscle but did not interfere with the formation of hexosephosphoric acid. From muscle juice containing fluoride Embden and Zimmerman isolated hexosediphosphoric acid identical with the fermentation acid. Working without fluoride they obtained only hexosemonophosphoric acid (lactacidogen). The monophosphate was converted by muscle juice to lactic and phosphoric acids, and in presence of fluoride to hexosediphosphoric acid. Pryde and Waters confirmed these results and showed that 90 per cent of the monoester was *d*-glucose monophosphate.

Lohmann showed that part of the ester accumulated under these conditions could not be the true Harden and Young diphosphoric acid, since with HCl at 100° it splits off phosphoric acid much more slowly than does that ester. In 1933 Embden showed that under Lohmann's conditions phosphoglyceric acid is formed, and transformed by minced muscle into pyruvic acid.²⁷ He suspected that phosphoglycerol must appear at the same time as the reduction product, and he suggested the following scheme for conversion of hexosediphosphate to lactic acid. Fructosediphosphate is first converted to a mixture of the monophosphates of glyceraldehyde and dihydroxyacetone which pass to a mixture of phosphoglycerol and phosphoglyceric acids. Phosphoglyceric acid is then converted to pyruvic and phosphoric acids, the pyruvic acid reacts with phosphoglycerol to give lactic acid and a molecule of triosephosphate, which passes to a mixture of phosphoglycerol and phosphoglyceric acids and the cycle is repeated. Meyerhof and Kiesel actually isolated phosphoglycerol. Addition of either this or pyruvic acid to carbohydrate free muscle extract does not give lactic acid, but addition of both simultaneously gives 2 molecules of lactic acid per molecule of pyruvic acid disappearing.



The phosphoglycerol is the *l*-variety and only this and not the optical isomer forms lactic from pyruvic acid. According to Embden's scheme pyruvic acid and phosphoglycerol give lactic and triosephosphoric acids, the latter rearranging to a mixture of phosphoglycerol and

phosphoglyceric acids and the cycle of reactions recurring until the whole is converted to lactic acid. This can be proved by addition of NaF which inhibits the splitting off of H_3PO_4 from phosphoglyceric acid when the reaction becomes



Under these conditions the pyruvic acid disappearing is equivalent to the lactic acid formed. No phosphate is split off and the phosphoglycerol is converted into phosphoglyceric acid. Addition of iodoacetic acid on the other hand does not inhibit the separation of H_3PO_4 from phosphoglyceric acid but prevents the formation of lactic acid by reaction of pyruvic acid and phosphoglycerol.

Glyceraldehydophosphoric acid, the probable intermediate triose-phosphate, was recently synthesized by Fischer. Smythe and Gerischer showed that the dextrorotatory component was easily fermented and Embden obtained from it lactic acid on treatment with muscle tissue. Meyerhof showed that exactly half of the synthetic acid (one optical component) is transformed by muscle extracts into a mixture of phosphoglyceric acid and phosphoglycerol. With added sulfite, but without fluoride, pyruvic acid and phosphoglycerol were formed, the glyceraldehydophosphoric acid thus acting exactly as does the intermediate product.

In the reactions of his fermentation scheme, Neuberg assumed that pyruvic acid was an intermediate substance giving acetaldehyde under the action of carboxylase and it was wrongly assumed that the acid arose from methylglyoxal. Neuberg has now shown with dried yeast that phosphoglyceric acid can be transformed into pyruvic acid. In fresh yeast extracts therefore phosphoglyceric acid will be readily decomposed into acetaldehyde and CO_2 . As early as 1929 Nilsson isolated phosphoglyceric acid from dry yeast in presence of fluoride, hexosediphosphate and acetaldehyde. In maceration juice containing fluoride, hexosediphosphoric acid is converted into Lohmann's "unhydrolyzable ester," consisting of equimolecular portions of phosphoglycerol and phosphoglyceric acids. The phosphoglyceric acid with fresh yeast extract gives CO_2 , H_3PO_4 and acetaldehyde, but phosphoglycerol is not further attacked and cannot therefore be formed during normal fermentation, and aldehyde must therefore be reduced not by it but by some other substance.

Addition to maceration extract containing fluoride, of glucose alone does not lead to esterification, of hexosediphosphate leads to the formation of phosphoglyceric acid and phosphoglycerol, of glucose and hexosediphosphate results in a quantity of glucose, equivalent to the hexosediphosphate being converted to phosphoglycerol and phosphoglyceric acids. Addition also of acetaldehyde, however, alters the

whole system. The aldehyde is converted to alcohol, the glucose esterified is converted solely to phosphoglyceric acid, phosphoglycerol appearing only to the extent that hexosediphosphate is decomposed. The esterification and oxidation of the glucose proceeds until all the aldehyde has been converted into alcohol. Since the reaction depends only on the amount of aldehyde added, the concentration of hexosephosphate can be reduced very considerably, less than 0.1 mg. per cc. of the fermentation mixture esterifying 50 or 100 times as much glucose. This small amount which acts as a catalyst is the same as that required in maceration extracts to initiate fermentation and to abolish the induction period. Glyceraldehydephosphoric acid like hexosediphosphate abolishes the induction period, and there appears to be nothing to contradict the supposition that it is the intermediate product. Acetaldehyde, which oxidizes the momentary reaction products can be replaced by other reducible systems; *e. g.*, methylene blue; and in this case also the oxidation product appears to be phosphoglyceric acid. The aldehyde reacts then with a nascent triosephosphoric acid (glyceraldehyde phosphoric acid), produced from the reaction of glucose with hexosediphosphate, and at the same time prevents the rearrangement of the triosephosphoric acid into phosphoglycerol so that the triosephosphoric acid is entirely oxidized to phosphoglyceric acid.

In muscle extract, therefore, pyruvic acid is reduced to lactic acid by phosphoglycerol, whereas in yeast fermentations it is split up into CO_2 and acetaldehyde which is then reduced not by phosphoglycerol but by some precursor, probably glyceraldehydephosphoric acid.

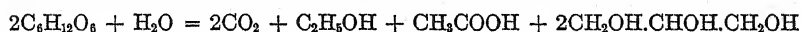
(a) **Alcoholic Fermentation** (continued).—2. *Modified*.—(a) *By Sulfites*.—The classical equation $\text{C}_6\text{H}_{12}\text{O}_6 = 2\text{CO}_2 + 2\text{C}_2\text{H}_5\text{OH}$ is regarded by Neuberg as expressing the first form of the ordinary alcoholic equation. It was found by Neuberg and Reinfurth and by Connstein and Lüdecke that when Na_2SO_3 is added to a fermenting mixture of yeast and sugar the yields of alcohol and CO_2 decrease while those of glycerol (normally 2–3 per cent of the sugar fermented) and acetaldehyde (as the nonreducible bisulfite compound) increase considerably. The latter authors employed the method during the war for the preparation of 1,000,000 kilos of glycerol per month, the recovery yield being 15–20 per cent of the sugar fermented. Neuberg and Reinfurth found that the amount of aldehyde produced was exactly equivalent to the glycerol and Neuberg and Hirsch showed that this relationship held during the whole fermentation.

In presence of sulfite, Neuberg expresses this second form of fermentation by the equation $\text{C}_6\text{H}_{12}\text{O}_6 + \text{Na}_2\text{SO}_3 + \text{H}_2\text{O} = \text{C}_3\text{H}_8\text{O}_3 + \text{C}_2\text{H}_4\text{O} \cdot \text{NaHSO}_3 + \text{NaHCO}_3$. The equation is never fully realized since the bisulfite component is to some extent dissociated and the aldehyde attacked and some sugar always undergoes the normal fermentation. In this modified reaction, the hydrogen which is normally accepted by aldehyde is accepted by some unknown substance with the production of glycerol. The amounts of glycerol and aldehyde obtained

increase with the amount of sulfite up to certain limits when the action of the yeast becomes inhibited. Neuberg and Reinfurth obtained similar results with the sulfites of Ca, Mg and Zn. The bisulfite compound of glucose is largely hydrolyzed in aqueous solution and the sugar fermented, and that of pyruvic acid, although stable, is readily fermented. The fixation method works best with living yeast but also succeeds with yeast preparations. In experiments in the presence of sulfites, the velocity constants of the first and second forms of fermentation are normally equal and it appears certain that the production of aldehyde is part of the normal reaction and not the result of a secondary reaction due to the presence of Na_2SO_3 .

(b) *By Dimedone*.—Neuberg and Reinfurth found that dimedone (dimethylcyclohexanedione), which combines with acetaldehyde but not with glucose or pyruvic acid, exerts the same influence on the course of fermentation as sulfite, the aldehyde being converted into anhydro-acetaldehyde-bis-dimethylcyclohexanedione. The reaction proves conclusively that the reaction is due to the specific power of combining with aldehyde and is not due to the alkalinity of the reagent as was possible with sulfite.

(c) *By Sodium Carbonate*.—Alkalis also modify the course of alcoholic fermentation but in a somewhat different manner from sulfites. Thus in presence of alkaline carbonates and a variety of other alkaline substances Neuberg, Hirsch, Reinfurth and Ursum showed that the aldehyde undergoes a Cannizzaro reaction giving rise to equimolecular proportions of acetic acid and alcohol. The removal of aldehyde involves the production of a molecular equivalent of glycerol, the full equation for this third form of fermentation being therefore



(d) *By Active Charcoal*.—Abderhalden and his colleagues found that animal charcoal increases the rate of fermentation of glucose, the smell of acetaldehyde being readily detectable. Addition of pyruvic acid, acetaldehyde, methyl or ethyl alcohols to a charcoal yeast mixture always leads to the formation of CO_2 and with pyruvic acid to the liberation also of acetaldehyde. Removal of acetaldehyde from the fermenting mixture by adsorption on the charcoal increases the amount of available hydrogen and so increases the amount of glycerol from 2–3 per cent to 7–8 per cent of theory. The increased fermentation rate is regarded as due partly to the increased formation of acetaldehyde and partly to adsorption of alcohol.

(e) *Phytochemical Reduction*.—The reduction during yeast fermentations of acetaldehyde to ethyl alcohol and also of some precursor, probably glyceraldehyde, to glycerol has already been discussed. Many other reductions have been investigated particularly by Neuberg and his collaborators, and a few will be outlined here. Thus Neuberg and Walde showed that fermenting yeast converts PhNO_2 into a 70 per cent yield of PhNH_2 , EtNO_2 and MeNO_2 into the corresponding

amines, PhCHO into a 22–32 per cent yield of PhCH_2OH (here only a trace of PhCOOH was obtained and the alcohol cannot possibly be formed by a Cannizzaro reaction) and PhCH_2CHO (12 Gm.) into $\text{PhCH}_2\text{CH}_2\text{OH}$ (7.5–8.5 Gm.). Neuberg and Steenbock obtained from commercial valeraldehyde with a yeast sugar fermentation 60–84 per cent of amyl alcohol, with yeast in the absence of sugar 17 per cent, and with cell-free material 12 per cent of amyl alcohol, while Neuberg and Nord obtained from 22.2 Gm. of $\text{C}_4\text{H}_9\text{CHO}$ 15 Gm. of *n*-amyl alcohol. More recently Friedmann has studied the reduction of acetoacetic acid to β -hydroxybutyric acid in presence and absence of glucose and Fujise the reduction of oxalacetic ester to *l*-malic acid.

Finally the production of optically active glycols by fermenting yeast was first accomplished by Neuberg and his collaborators. Thus Neuberg and Kerb converted acetaldol into β -butyleneglycol, Neuberg and Nord reduced various diketones and Färber, Nord and Neuberg reduced acetol to propyleneglycol. The method has been extensively used by Levene, Walti and Haller while Levene and Walti have investigated the constitution of the glycols.

II. ACTION OF YEASTS ON NITROGENOUS SUBSTRATES

Production of Higher Alcohols: Fusel Oils.—Fusel oil, the high-boiling fraction of all forms of fermentation alcohol, amounts to 0.1–0.7 per cent of the distilled crude spirit and consists chiefly of *iso*amyl and *d*-amyl alcohols with much smaller amounts of propyl and isobutyl alcohols and traces of fatty acids, aldehydes, etc. Ehrlich²⁰ and his co-workers were the first to show conclusively that these substances are derived from amino acids formed by hydrolysis of proteins. These workers first showed that protein on hydrolysis yields besides leucine the isomeric isoleucine. When leucine and isoleucine were added to a sugar solution and a pure culture of yeast introduced, they readily gave rise respectively to *iso*amyl and *d*-amyl alcohols, the yields being 87 and 80 per cent of theoretical. This change brought about by living yeast does not occur when zymine or yeast juice are used, and the reaction appears to be intimately connected with the nitrogenous metabolism of the cell, and is a general one for α -amino acids. Thus tyrosine yields *p*-hydroxyphenylethyl alcohol, a bitter substance which assists in determining the flavor of beers, phenylalanine yields phenylethyl alcohol, one of the constituents of oil of roses, and tryptophan yields tryptophol. It will be seen therefore that the amounts of the alcohols formed during fermentation will depend on the amino-acid content of the proteins contained in the various media used (molasses, corn, potatoes, grapes, etc.). Yeast also forms fusel oil at the expense of its own protein especially when the supply of nitrogen is deficient. During the fermentation of the racemic form of these amino-acids, both optical isomers are attacked but at unequal rates. Thus with *dl*-leucine, *l*-leucine completely disappears and *d*-leucine may at one stage be isolated in the pure state.

METABOLISM OF LOWER FUNGI

The widespread interest in the growth and metabolism of the lower fungi or "molds" has recently received great impetus both from a recognition of their manifold synthetic activities and from their recent employment in fermentation industries.

Much of the earlier work on the metabolism of these micro-organisms has already been reviewed by Raistrick³¹ and it is our purpose to review briefly the effect of nutritional factors on their growth and also to extend the previous article so as to include the most recent results.

I. ACTION OF LOWER FUNGI ON NONNITROGENOUS SUBSTRATES

Molds are able to synthesize their cell substances from the most diverse sources of carbon. Thus Tamiya investigated the growth of *Aspergillus oryzae* on 123 pure organic substances. The best sources of carbon are the carbohydrates and polyatomic alcohols (glycerol, mannitol, inositol), the alcohols of the aromatic series not being utilized. Succinic, lactic, malic, citric and salicylic acids are assimilated, pyruvic acid is utilized more readily than lactic, lactic more than propionic, succinic more than itaconic and fumaric more than mesaconic acid. Most sources of carbon are, however, utilized by some specific group of organisms. Thus Tausson showed that *A. flavus* utilizes both paraffins and waxes, while Hopkins and Chibnall found that *A. versicolor* grows on higher paraffins with chains not exceeding $C_{34}H_{70}$.

The alleged parallelism between the production of mycelial tissue by *A. niger* and the supply of certain mineral substances has led to the utilization of the organism as an indicator of the nutrient value of soils. According to Rippel and Stoess, calcium is not essential for the development of these organisms but it does increase growth in certain cases; *e. g.*, *Rhizopus*, *Penicillium* and *Fusarium*, and it appears to antagonize magnesium. On the other hand the mycelium of *A. niger* grown under conditions which make the supply of potassium a limiting factor contains 0.12 per cent K_2O and the production of mycelium is proportional to the amount of the element available. The same holds for magnesium. *A. niger* is able to utilize the soluble alkali and calcium phosphates and also the insoluble Ca and Fe phosphates and the phosphorus of phytin. The effect of heavy metal salts has had a very obvious bearing on the development of such industrial processes as the manufacture of citric acid.

(a) **Production of Oxalic, Citric, Succinic, Fumaric, Gluconic Acids, etc.**—*Oxalic acid* was first recognized by Wehmer as a definite fermentation product. He obtained it by growth of *A. niger* on a variety of substrates and found that by addition of $CaCO_3$ to the medium, large yields of calcium oxalate may be obtained. Other observers have since confirmed his results; *e. g.*, Heinze, Emmerling, Currie and Thom,³² Elfving, Raistrick and Clark. Currie and Thom obtained this acid using 17 strains of *A. niger* and also *A. ochraceus*, *A. violaceofuscus* Gasperini and *Penicillium oxalicum* Currie and Thom.

Citric acid was also obtained by Wehmer first using *Citromyces glaber* and *C. Pfefferianum* and later using *Penicillium luteum*. More recent work has shown that the acid is a fairly common metabolic product of many different species belonging to different genera. Thus Currie showed that with the proper adjustment of experimental conditions, the production by *A. niger* of oxalic acid can be entirely suppressed and large yields of citric acid obtained. The process has for many years been worked in America for the large scale manufacture of this acid. Among many other papers dealing with the production of citric acid from different substrates, the following are chosen in order to show the wide range of species producing this acid: From *P. glaucum* and *Citromyces glaber*, from 43 species of *Penicillium*,³³ from *P. divaricatum* and *P. citrinum*, from *A. parasiticus* Speare and *P. spinulosum*.³⁰

d-Gluconic acid was first obtained by Molliard using *A. niger* and later by various workers, e. g., Bernhauer using *A. niger*,³⁴ Butkewitsch using *Citromyces glaber* and *P. glaucum*, Falck and Kapur using *A. niger*, *fuscus* and *cinnamomeus*, and Birkinshaw and Raistrick using *P. chrysogenum* and *Fumago vagans*.³⁰ Under optimal conditions Herrick and May obtained 80 per cent yields of *d*-gluconic acid and give details for its preparation in quantity.

d-Mannonic acid was obtained by Angeletti and Cerruti by growing *P. purpurogenum* var. *rubrisclerotium* on a synthetic medium containing *d*-mannose, the yield being 9 per cent.

Fumaric acid is not a common mold-metabolic product. It was first obtained in small yield by Ehrlich using *Rhizopus nigricans* and later Wehmer described a new species, *A. fumaricus* which gave large yields of this acid. The characteristic was, however, gradually lost and replaced by the production of gluconic acid. Recently Raistrick and Simonart,³⁹ using *P. griseo-fulvum* Dierckx, also isolated small amounts of fumaric acid.

Malic acid was isolated in small yields by Wehmer using *A. fumaricus* and by Raistrick *et al.*³⁰ using a white species of *Aspergillus* and a species of *Clasterosporium*.

Succinic acid has been obtained by Fitz using *Mucor mucedo*, by Emmerling using *M. racemosus*, by Sumiki using *Oidium lupuli*, by Raistrick *et al.*³⁰ using a white species of *Aspergillus*, a species of *Clasterosporium*, *Fumago vagans* and *P. spiculisporum*. It is not known whether the acid arises as a direct product of the oxidative breakdown of sugar or whether it arises, as is believed to be the case in alcoholic fermentation, from the breakdown of the cell proteins.

Itaconic acid has recently been obtained by Kinoshita³⁵ using an organism, *A. itaconicus*, found under natural conditions in the expressed sap of pickled plums.

Ethyl alcohol is produced by various species of *Mucor* and *Rhizopus* while Raistrick *et al.*³⁰ showed that 23 species of *Fusarium* all gave alcohol from glucose, differences being only quantitative. The latter

authors also investigated the production of alcohol by 96 species or strains of *Aspergillus* and 75 species of *Penicillium*. They found that while ethyl alcohol is a frequent metabolic product of species belonging to these two genera, it is not a constant product as with *Fusaria*. Thus while it was produced by all the 7 species examined in the *A. flavus-oryzae-tamaris* group, it was entirely absent from the products of 9 species in the *A. glaucus* group. Finally these authors also obtained ethyl alcohol using *Eidamia viridescens*, *Eidamia catenulata*, *Trichoderma lignorum* and *Helminthosporium geniculatum*.

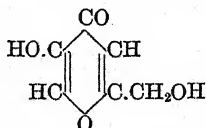
Acetaldehyde.—Cohen and Neuberg found that acetaldehyde was produced from glucose and sucrose by species of *Monilia*, *Mucor*, *Oidium*, *Aspergillus* and *Penicillium*, the aldehyde being fixed by the sulfite method. Birkinshaw and Raistrick obtained small amounts of acetaldehyde using *Helminthosporium geniculatum* and a species of *Clasterosporium* without any interceptor present.

Molds do not in general produce any large amounts of volatile acids although Butkewitsch and Fedoroff believe that the formation of succinic acid and fumaric acid by *Rhizopus nigricans* when grown on alcoholic solutions takes place by way of acetic acid, and Birkinshaw, Charles and Raistrick showed that *P. digitatum* Saccardo (*P. olivaceum* Wehmer), a species of penicillium responsible for the rot of citrous fruits, when grown on the usual Czapek-Dox glucose medium, gives rise to considerable amounts of ethyl acetate.

(b) **Production of Glycerol and Mannitol.**—*Glycerol* was found as a metabolic product of the growth on sucrose of *Mucor racemosus* by Emmerling and of *A. niger* by Molliard. Raistrick *et al.*³⁰ also obtained it from glucose using a white species of *Aspergillus*, *Helminthosporium geniculatum*, *Clasterosporium* species and *A. Wentii*.

Mannitol along with trehalose was obtained in the mycelium of *A. niger*. Obaton found that with a glucose medium a large yield of trehalose was obtained and little mannitol, whereas with invert sugar or fructose the reverse proportions were produced. Raistrick *et al.* investigated the production of mannitol by 3 white species of *Aspergillus*, *A. elegans*, 5 strains of *A. nidulans*, *A. Wentii*, *P. chrysogenum*, *Helminthosporium geniculatum* and a species of *Clasterosporium*. With a white species of *Aspergillus*, mannitol yields of 50 per cent of the sugar utilized were obtained, and Coyne and Raistrick found that mannitol was produced from glucose, mannose, galactose, xylose and arabinose. Fructose did not give mannitol, the picture being the reverse of that with bacterial fermentation.

(c) **Production of Special Products.**—1. *Kojic acid*,



was first detected by Saito using *A. oryzae*, and subsequently isolated

and its constitution elaborated by Yabuta,³⁶ using *A. oryzae*, *albus*, *candidus* and *nidulans*. It has since been obtained by numbers of workers. Challenger, Klein and Walker obtained it using *A. oryzae* from xylose and arabinose while Katagiri and Kitahara obtained yields of 10–40 per cent from glucose, maltose, sucrose, inulin, fructose and xylose and of 1–6 per cent from mannose, arabinose and galactose. May, Moyer, Wells and Herrick worked out the conditions for *A. flavus* for the optimum production of kojiic acid with a view to possible commercial exploitation. Raistrick *et al.*³⁰ found that when the composition of medium, temperature of incubation, etc., are rigidly controlled, the production of this acid may be used as a diagnostic test for species belonging to the *A. flavus-oryzae-tamarii* group.

2. *6-Hydroxy-2-methylbenzoic acid*, was obtained in 2.5 per cent yield by Anslow and Raistrick³⁸ using *P. griseo-fulvum* Biourge and a Czapek-Dox medium containing glucose as the sole source of carbon.

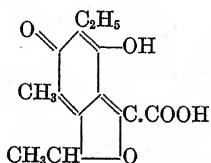
3. *Gentisic acid*, 2:5-dihydroxybenzoic acid, has been isolated by Raistrick and Simonart³⁹ as a product of the metabolism of glucose by *P. griseo-fulvum* Dierckx.

4. *Penicillic acid*, $C_8H_{10}O_4$, was described by Alsberg and Black³⁷ as a metabolic product of *P. puberulum* Bainier. It is toxic to animals when injected in a dosage of 0.2–0.3 Gm. per kilo body weight.

5. *Methoxydihydroxy-p-toluquinone*, $C_8H_8O_5$ was isolated by Birkinshaw and Raistrick³⁰ using 3 strains belonging to Thom's *P. spinulosum* series. In cultures on Czapek-Dox glucose medium, this quinone imparts a characteristic purple color.

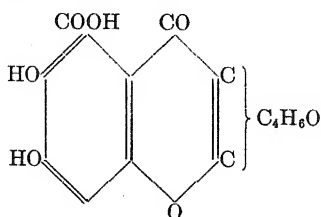
6. *Puberulic acid*, $C_8H_6O_6$, and an acid $C_8H_4O_6$ have been isolated by Birkinshaw and Raistrick⁴⁰ using *P. puberulum* Bainier and *P. aurantio-virens* Biourge on a glucose medium. The constitution of puberulic acid is not yet elucidated but it is not a dihydroxybenzene dicarboxylic acid.

7. *Citrinin*, $C_{13}H_{14}O_5$, was first isolated by Hetherington and Raistrick in 5 per cent yield using *P. citrinum* Thom on a slightly modified Czapek-Dox glucose medium. From a consideration of various degradation products, Coyne, Robinson and Raistrick assigned the accompanying formula. Citrinin is readily reduced by nascent



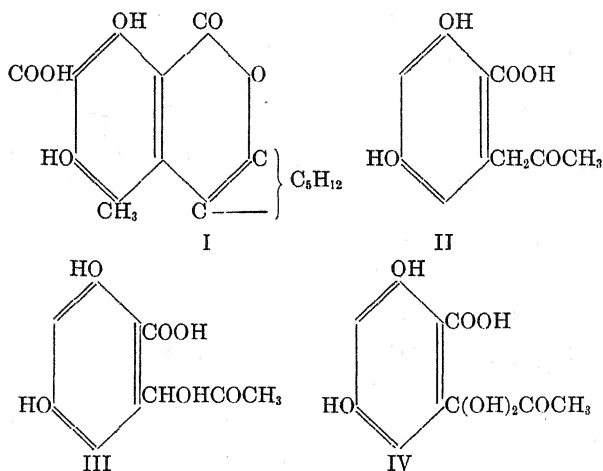
hydrogen and re-oxidized by atmospheric oxygen.

8. *Citromycetin*, $C_{14}H_{10}O_7 \cdot 2H_2O$, was obtained by Hetherington and Raistrick using *P. (Citromyces) glabrum* Wehmer, and the accompanying formula assigned. Citromycetin is more typical of certain



species of citromyces than is citric acid since of several hundred species of different genera, only a few species of *Citromyces* produce it or give its typical color reactions.

9. *Mycophenolic Acid*, $\text{C}_{17}\text{H}_{20}\text{O}_6$, and the *Accompanying Acids*, $\text{C}_{10}\text{H}_{10}\text{O}_5$, $\text{C}_{10}\text{H}_{10}\text{O}_6$, $\text{C}_{10}\text{H}_{10}\text{O}_7$.—The production of these substances from glucose by *P. brevi-compactum* Dierckx (syn. *P. stoloniferum* Thom) has been studied by Clutterbuck, Oxford, Raistrick and Smith.⁴¹



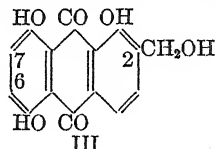
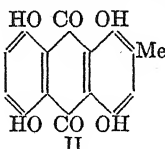
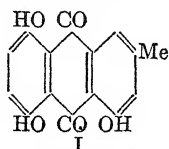
Mycophenolic acid was first isolated by Alsberg and Black³⁷ and it has been shown to be the monomethyl ether of I by Clutterbuck and Raistrick,⁴² while the C_{10} acids have been shown to have the constitutions II, III and IV.⁴³ The order of formation of these substances has also been investigated by Oxford and Raistrick.⁴³ In this work 15 strains of *P. brevi-compactum* were used and it was found that cultures freshly isolated from spoiled maize gave large yields of mycophenolic acid whereas cultures which had been maintained for a long time (twenty years) under artificial conditions no longer gave this acid but gave large yields of the C_{10} acids.

10. *3:5-Dihydroxyphthalic acid* was also isolated by Oxford and Raistrick⁴⁴ as a product of the metabolism of *P. brevi-compactum* Dierckx in the above experiment.

11. *Monascorubrin*, $\text{C}_{22}\text{H}_{24}\text{O}_5$, and *monascoflavin*, $\text{C}_{17}\text{H}_{22}\text{O}_4$,

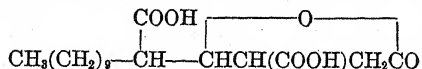
a red and yellow pigment, have recently been isolated from the mycelium of *Monascus purpureus* Went by Nishikawa, while Salomon and Karrer report the formation by *M. purpureus* on rice of a yellow pigment monascin, $C_{24}H_{30}O_6$. The constitution of these substances has not yet been reported.

12. *Oxyanthraquinones produced by species of Helminthosporium.*



Charles, Raistrick, Robinson and Todd⁴⁵ found that the dried mycelium of *Helminthosporium gramineum* Rabenhorst, a plant pathogen and causative agent of the well-known "leaf stripe" disease of barley, when grown on a synthetic medium containing glucose contained 30 per cent of oxyanthraquinones consisting of a mixture of helminthosporin, $C_{15}H_{10}O_5$ (I) and catenarin, $C_{15}H_{10}O_6$ (III). *H. cynodontis* Marignoni gave small amounts of helminthosporin with considerable amounts of cynodontin, $C_{15}H_{10}O_6$ (II).⁴⁶ The same authors have obtained considerable yields of catenarin using *H. catenarium* Drechsler and *H. tritici-vulgaris* Nisikado, catenarin together with ergosterol using *H. velutinum* Link, and cynodontin using *H. avenae* Eidam. Evidence by color reactions of the production of smaller amounts of oxyanthraquinones was also obtained using 10 other species of *Helminthosporium*, while no such reaction was obtained with 16 other species. The constitutions of cynodontin and helminthosporin have been satisfactorily elucidated by the same authors, and that of helminthosporin confirmed by synthesis. The constitution of catenarin (III) is not yet fully ascertained, the position of the CH_2OH group being either as shown in position 2 or in positions 6 or 7.

13. *Spiculisporic acid*, $C_{17}H_{28}O_6$, was first isolated by Clutterbuck, Raistrick and Rintoul⁵⁰ using *P. spiculisporum* Lehman and was shown to be the lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecic acid.



The acid has since been obtained by Birkinshaw and Raistrick working with *P. minioluteum*.

14. γ -Ketopentadecic acid, $C_{15}H_{28}O_3$, was also obtained by the same authors along with spiculisporic acid using *P. spiculisporum* Lehman.

15. *Minioluteic acid*, $C_{16}H_{26}O_7$, was first isolated by Birkinshaw and Raistrick and is closely related to spiculisporic acid.

16. *Glauconic acid I*, $C_{18}H_{20}O_7$, and *glauconic acid II*, $C_{18}H_{20}O_6$, were first isolated by Wijkman using a strain of *P. glaucum*, and Sutter and Wijkman⁴⁷ have begun a study of their constitution. An

acid probably identical with gluconic acid I has been obtained by Yuill using a strain of *P. luteum-purpurogenum* occurring as a parasite on *A. niger*.

17. *Byssochlamic acid*, $C_{18}H_{20}O_6$, was obtained by Raistrick and Smith using *Byssochlamys fulva*. The acid is certainly not identical with gluconic acid II.

18. *Carolic and Related Acids*.—Clutterbuck, Haworth, Raistrick, Smith and Stacey⁴⁸ using *P. Charlesii* G. Smith have obtained the following series of related acids: Verticillic $C_{26}H_{32}O_{12}$, ramigenic $C_{16}H_{20}O_6$, carlic $C_{10}H_{10}O_6$, carlosic $C_{10}H_{12}O_6$, carolinic $C_9H_{12}O_7$, and carolic $C_9H_{10}O_4$. Carolic acid on hydrolysis with dilute boiling acid gives 1 molecule each of CO_2 , butyrolactone and acetoin and it is certain that these acids do not possess a benzenoid structure.

19. *Pigments from Species of the A. Glaucus Series*.—Using species of the *A. glaucus* series, Gould and Raistrick have isolated in the pure state red, yellow and orange pigments, having the empirical formulae respectively of $C_{16}H_{12}O_5$ (probably a hydroxyanthraquinone), $C_{19}H_{28}O_3$ and $C_{19}H_{22}O_3$.

20. *Polysaccharides*.—"Spore" or "mold" starch, giving a blue color with iodine, was early obtained by Cramer and Wehmer using *A. niger*, *A. fumigatus* and *P. variabile*. Boas examined it in detail obtaining it from various sugars, mannitol, glycerol, malic, tartaric, citric and oxalic acids using *A. niger*, *oryzae* and *glaucus*. Lappalainen, working with *A. niger*, investigated the effect of temperature and concentration of nutrients in the medium on the production of starch. Chrzaszcz and Tiukow,³³ working with 45 species of *Penicillium* and *Citromyces*, state that these may be divided into two groups, the starch formers and the acid formers, starch or excess of acid filling the rôle of reserve material. Alsberg and Black³⁷ obtained a similar substance from the mycelium of *P. puberulum* which gave an intense violet color with iodine and was considered to be identical with trehalum from manna. Dox and Neidig isolated from the mycelium of *P. expansum* a polysaccharide to which they gave the name of mycodextran. This substance gave no color with iodine and gave glucose as the sole product of hydrolysis. The same authors using *A. niger* obtained besides mycodextran, mycogalactan which gave no color with iodine and only galactose on hydrolysis.

Birkinshaw, Charles, Hetherington and Raistrick³⁰ describe the production by a white species of *Aspergillus* of a polysaccharide either identical with or closely allied to glycogen. Two other polysaccharides, both giving only glucose on hydrolysis, and neither giving any color with iodine were obtained by Birkinshaw and Raistrick³⁰ using *Fumago vagans*, and by Birkinshaw, Charles and Raistrick³⁰ using three different strains of *P. digitatum* Saccardo. A polysaccharide of particular interest was obtained by Haworth, Raistrick and Stacey by growing *P. varians* on a Czapek-Dox glucose medium. The homogeneous polysaccharide was shown to contain units of glucose, galactose and a third

sugar which proved to be either *d*-idose or *l*-altrose. Working with *P. Charlesii* G. Smith and a glucose-salts medium, Clutterbuck; Haworth, Raistrick, Smith and Stacey⁴⁸ isolated a polygalactose and a polymannose.

All the above carbohydrates have been neutral in reaction. A different type of product, to which the name luteic acid was given, was described by Raistrick and Rintoul.³⁰ This substance, which was obtained in yields of 10–12 per cent, was produced by a nonascosporic form of *P. luteum* Zukal when grown on a Czapek-Dox glucose medium. Luteic acid is a mucilaginous material which on hydrolysis gives rise to glucose and malonic acid in the proportions of 2 molecules of the former to one of the latter. On hydrolysis with dilute barium hydroxide it yields a neutral polyglucose, luteose. Luteose and luteic acid give no color with iodine. Using the same organism, Birkinshaw and Raistrick⁴⁰ found that luteic acid is elaborated when the medium contains as the sole source of carbon any of the following substances: Glucose, fructose, galactose, mannose, xylose, arabinose, and glycerol. Indications of a similar product were obtained with succinic and citric acids as substrates.

21. *Fatty Substances*.—(a) *Neutral Fats*.—Much of the early work on the production of fats by molds may be omitted since the cultures used were of doubtful authenticity and purity. In 1921 a German patent was taken out for the production of fat by inoculating turnips, apples, etc., with certain specified fungi, and a little later Belin using *A. niger* and Barber using an unnamed species of *Penicillium* reported the formation of fat. Terroine next concerned himself with the formation of fat by *A. niger* from sugars, chiefly from the energy standpoint. Pearson and Raper showed that the iodine value of the fatty acids produced by *A. niger* and *Rhizopus nigricans* varies with the temperature at which these are grown, the acids being more saturated at the higher temperature, this being in conformity with the observation that, in general, the more saturated fats found in nature are usually produced at higher temperatures than the less saturated ones. Thom in his book on "The Penicillia" mentions that among his own cultures, certain strains with the aspect of Corda's *Clonostachys araucaria* produced masses of mycelium apparently full of fat globules. Charles, Raistrick, Robinson and Todd⁴⁵ in the preparation of their oxyanthraquinones using species of *Helminthosporium* obtained along with the pigments large yields of fat of low iodine value.

(b) *Sterols*.—Gérard, working with *P. glaucum* on Raulin's medium obtained a sterol resembling ergosterol and later obtained a second sterol using *Mucor mucedo* and lactose. Gayral determined quantitatively the amounts of "phytostérine" produced by *A. niger* under the influence of different types of radiations. Reindel and Walter obtained a sterol using *Mucor* which was identical with ergosterol of yeast fat. The ergosterol content of *Dematium pullulans*, *P. glaucum* and *A. oryzae* was found by Heiduschka and Lindner to be 0.30, 0.75 and 0.46

per cent of the dry matter of these fungi. Sumi isolated 0.8 Gm. of crude ergosterol, from 1 Kg. *A. oryzae* spores, while Takata obtained 2.8 Gm. crude sterol, giving the characteristic reactions for ergosterol, from 1 Kg. of the dried mycelium of the same organism.

Prickett, Massengale, Cox and Bills studied the factors affecting the ergosterol content of 18 molds and Pruess, Peterson, Steenbock and Fred, in a paper on the "Sterol content and antirachitic activatability of mold mycelia" investigated the growth and sterol production of a large number of the lower fungi. Distinct healing of rickets was effected on feeding to rachitic rats of irradiated autoclaved dried mycelia of 8 of the species tested. Birkinshaw, Callow and Fischmann⁴⁰ obtained a sterol using *P. puberulum* Bainier and found that the physical properties of the sterol itself and of its benzoate corresponded with those of ergosterol and ergosterol benzoate, and confirmed their findings by measurements of the absorption spectrum and of the antirachitic activity acquired after irradiation. Oxford and Raistrick⁴³ isolated ergosterol palmitate from the mycelia of 14 strains of *P. brevicompactum* Dierckx and also from the mycelium of *P. italicum* Wehmer. The mycelium of *P. aurantio-griseum* Dierckx var. *Poznanienensis* Zaleski contained 0.5 per cent of this ester.

Finally Pruess *et al.* have shown that with 30 species of *Aspergillus*, 20 of *Penicillium* and 15 species of other molds the nature of the medium is of great importance in the accumulation of sterols. Conditions favoring growth promoted the synthesis of sterols.

II. ACTION OF LOWER FUNGI ON NITROGENOUS SUBSTRATES

The ability of molds to fix atmospheric nitrogen has been repeatedly affirmed and denied by numerous investigators. The most recent positive data are given by Schober, who claims to have demonstrated that *A. niger* fixes nitrogen from the atmosphere, drawing the energy for its assimilation from the combustion of carbon compounds, chiefly glucose. Schröder repeated this work using the same strains, but could not confirm the conclusions, while Roberg tested 21 strains of *A. niger*, 5 of which had given positive results with Schober, using different media supplemented by various catalysts (Zn, Cu, Wo, Mo) but in no case was nitrogen fixation detected.

Working on the transformations of nitrogen, Sakamura found that *A. oryzae* can utilize nitrates, ammonium salts or both simultaneously. An excess of NH_4 salts results in the medium becoming acid and in growth being retarded, whereas absorption of nitrate does not change the pH materially and growth and nitrogen intake are high. Absorption of nitrate is favored by the presence of sugar. With ammonium nitrate, preferential absorption of ammonia causes the medium to become increasingly acid and to retard growth. Rippel found that nitrate is absorbed by *A. niger* after the ammonia has been used up.

Very little has been done in respect to the degradation of amino acids by molds, but the presence of proteases and other enzymes has

been detected. Thus Schmalfuss and Mothes obtained from *A. niger* an asparaginase which could be extracted from the mycelium with water or glycerol, and which converts asparagine into ammonium aspartate. Iwanoff and Awetissowa have shown that *A. niger* under certain conditions can assimilate guanidine, and contains a guanidinase which splits guanidine quantitatively into urea and ammonia.

In concluding this review, it is of interest to compare the metabolic products of molds and bacteria. Molds are essentially aerobic organisms and most of them possess such strong oxidative powers that, provided they are allowed sufficient air and a sufficiently long incubation period, they will oxidize completely to carbon dioxide and water, both the carbohydrate of the medium and the intermediate products of their own metabolism. The acids accumulating in mold cultures on carbohydrate media are invariably of the fixed type; *e. g.*, gluconic, citric, fumaric, oxalic acids, etc.; while in bacterial cultures volatile acids; *e. g.*, acetic, propionic, butyric and lactic acids are the most commonly occurring. Lactic acid has never been reported as a mold product but it is produced by large numbers of bacteria. On the other hand it must be remembered that according to Pfeffer acetic acid is utilized by *A. niger* in preference to glucose, and one would not therefore expect it to accumulate in the medium.

A further striking difference is the entire absence of hydrogen and methane as mold metabolic products which is in marked contrast to the frequent occurrence of these gases, particularly hydrogen, as products of bacterial metabolism. Ample evidence has been advanced in this review of the outstanding synthetic powers of the lower fungi, proof having been given of the production from carbohydrates of pyrone, benzpyrone, quinone derivatives, simple and complex phenolic acids, oxyanthraquinones, complex carbohydrates and fatty acids, a variety of lactonic acids, fats, sterols, etc. The function of these substances is largely a matter for future research, but it appears that some substances, *e. g.*, carbohydrates, fats and possibly some of the acids (gluconic, citric acids) may be regarded as reserve or storage products; others (citrinin) may form oxidation-reduction systems, while in other cases there appears to be definite evidence that the specific metabolic products form part of a defensive system elaborated by the organism to assist growth or to suppress the growth of other organisms. Thus Fleming and later Clutterbuck, Lovell and Raistrick⁵⁰ describe the production by a strain of *P. chrysogenum* Thom of a metabolic product at present not isolated but called by Fleming "penicillin." This substance has very marked bactericidal properties since it entirely suppresses growth of the pyogenic cocci and the diphtheria group of bacilli in very high dilutions although it is easily tolerated by many other bacteria, *e. g.*, the coli-typhoid group, the influenza-bacillus group and the enterococcus.

P. W. CLUTTERBUCK,
H. RAISTRICK.

REFERENCES

1. Thaysen, A. C., and Bunker, H. J.: *Microbiology of Cellulose*, etc. (1927).
2. Buchanan, R. E., and Fulmer, E. I.: *Physiology and Biochemistry of Bacteria* (1930).
3. Thaysen, A. C., and Galloway, L. D.: *Microbiology of Starches and Sugars* (1930).
4. Smyth, H. F., and Obold, W. L.: *Industrial Microbiology* (1930).
5. Stephenson, M.: *Bacterial Metabolism* (1930).
6. Stephenson, M.: *Annual Rev. Biochem.*, **1**, 2 (1932, 1933).
7. Van Niel, C.: *Propionic Acid Bacteria* (1928).
8. Bernhauer, K.: *Die Oxydativen Gärungen* (1932).
9. Kluwyer, A. J.: *Chemical Activities of Microorganisms* (1931).
10. Raistrick, H.: *Biochem. J.*, **11**, 71 (1917); **13**, 446 (1919).
11. Herrick, H. T., et al.: *Ind. and Eng. Chem.*, **22**, 1148 (1930).
12. Hopkins, F. G., and Cole, S. W.: *J. Physiol.*, **27**, 418 (1901); **29**, 451 (1903).
13. Birkinshaw, J. H., Charles, J. H. V., and Clutterbuck, P. W.: *Biochem. J.*, **25**, 1522 (1931).
14. Viljoen, J. A., Fred, and Peterson: *J. Agric. Sci.*, **16**, 1 (1926).
15. Reilly, J., et al.: *Biochem. J.*, **14**, 229 (1920).
16. Speakman, H. B.: *J. Biol. Chem.*, **41**, 319 (1920); **43**, 401 (1920); **64**, 41 (1925).
17. Peterson, W. H., Fred, E. B., and Anderson, J. A.: *J. Biol. Chem.*, **53**, 111 (1922).
18. Stiles, H. R., Peterson, W. H., and Fred, E. B.: *J. Biol. Chem.*, **64**, 643 (1925).
19. Heidelberger, M., and Goebel, W. F.: *J. Biol. Chem.*, **70**, 613 (1926); **74**, 613 (1927).
20. Ehrlich, F.: *Biochem. Z.*, **2**, 52 (1907); *Ber.*, **39**, 4072 (1906); *Ber.*, **40**, 1027 (1907).
21. Avery, O. T., and Goebel, W. F.: *J. Exp. Med.*, **58**, 731 (1933).
22. Raistrick, H., and Topley, W. W. C.: *Brit. J. Exp. Path.*, **15**, 113 (1934).
23. Harden, A.: *Alcoholic Fermentation* (1932).
24. Myrbäck, K., and Euler, H. v.: *Z. physiol. Chem.*, **136**, 107 (1924); **138**, 1 (1924); **139**, 281 (1924); **141**, 297 (1924); **176**, 258 (1928).
25. Robison, R.: *Biochem. J.*, **26**, 2191 (1932).
26. Neuberg, C., and Kobel, M.: *Biochem. Z.*, **203**, 463 (1928); **207**, 232 (1929).
27. Embden, G., Deuticke, H. J., and Kraft, G.: *Klin. Wochschr.*, **213** (1933).
28. Meyerhof, O.: *Nature*, **132**, 337, 373 (1933).
29. Meyerhof, O., and Kiessling, W.: *Biochem. Z.*, **267**, 313 (1934).
30. Raistrick, H., et al.: *Trans. Roy. Soc. London*, **220B** (1931).
31. Raistrick, H.: *Ergebnisse der Enzymforschung*, **1**, 345 (1932).
32. Currie, J. N., and Thom, C.: *J. Biol. Chem.*, **22**, 287 (1915).
33. Chrzaszcz, T., and Tiukow, D.: *Biochem. Z.*, **204**, 106 (1929); **207**, 39 (1929).
34. Bernhauer, K.: *Biochem. Z.*, **153**, 517 (1924); **172**, 313 (1926); **197**, 278, 287 (1928).
35. Kinoshita, R.: *J. Chem. Soc. (Japan)*, **50**, 583 (1929).
36. Yabuta, T.: *J. Chem. Soc.*, **125**, 575 (1924).
37. Alsberg, C. L., and Black, O. F.: *U. S. Dept. Agr. Bull.*, **270** (1913).
38. Anslow, W. K., and Raistrick, H.: *Biochem. J.*, **25**, 39 (1931).
39. Raistrick, H., and Simonart, P.: *Biochem. J.*, **27**, 628 (1933).
40. Birkinshaw, J. H., and Raistrick, H.: *Biochem. J.*, **26**, 441 (1932); **27**, 370 (1933).
41. Clutterbuck, P. W., Oxford, A. E., Raistrick, H., and Smith, G.: *Biochem. J.*, **26**, 1441 (1932).
42. Clutterbuck, P. W., and Raistrick, H.: *Biochem. J.*, **27**, 654 (1933).
43. Oxford, A. E., and Raistrick, H.: *Biochem. J.*, **27**, 634, 1473 (1933).
44. Oxford, A. E., and Raistrick, H.: *Biochem. J.*, **26**, 1902 (1932).
45. Charles, J. H. V., Raistrick, H., Robinson, R., and Todd, A. R.: *Biochem. J.*, **27**, 499 (1933).
46. Raistrick, H., Robinson, R., and Todd, A. R.: *Biochem. J.*, **27**, 1170 (1933).
47. Sutter, H., and Wijkman, N.: *Ann.*, **505**, 248 (1933).
48. Clutterbuck, P. W., Haworth, W. N., Raistrick, H., Smith, G., and Stacey, M.: *Biochem. J.*, **28**, 94 (1934).
49. Birkinshaw, J. H., Callow, R. K., and Fischmann, C. F.: *Biochem. J.*, **25**, 1977 (1931).
50. Clutterbuck, P. W., Lovell, R., and Raistrick, H.: *Biochem. J.*, **26**, 1907 (1932).

CHAPTER XIII

DETOXICATION

It has been necessary for the body to call to its aid a chemical defense mechanism to guard against the poisons absorbed from the gastro-intestinal tract. After many generations this chemical defense mechanism has been so perfected in its battle against putrefaction products absorbed from the intestine that it is now quite able to cope with many of the foreign organic compounds.

A study of these various chemical reactions is of interest because it affords a method of differentiating chemically between various types or species of animals.

From a standpoint of physiology it is very interesting to know which organs or tissues are capable of promoting each of these different reactions. From a pathologic standpoint it gives us an insight, qualitatively at least, regarding the amount of damage suffered by a certain organ when it is no longer able properly to function in promoting one of these defensive reactions. Clinically it is of importance if one can point out the organ involved and place the reactions on a quantitative basis; thus affording us a reliable index of organic functioning. From a standpoint of the dietitian it is also useful. For instance, the unessential amino acids of the protein molecule had been considered "unessential" because they could be excluded from the diet without harm to the subject. From a study of the detoxication reactions, it was found in some cases that the unessential amino acids were not necessary because they could be synthesized by the body, while the essential amino acid, cystine, could not be synthesized, regardless of the fact that the body was furnished available nitrogen and sulfur.

Again, a study of many of these reactions gives us an insight into the intermediary metabolism of the amino acids, the carbohydrates and perhaps the fats.

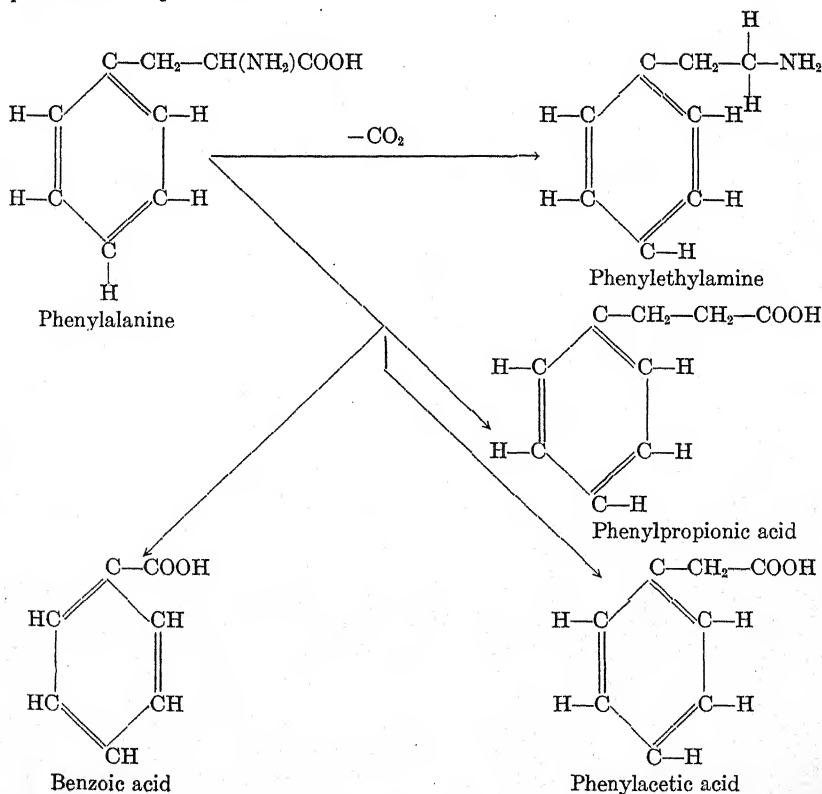
INTESTINAL PUTREFACTION

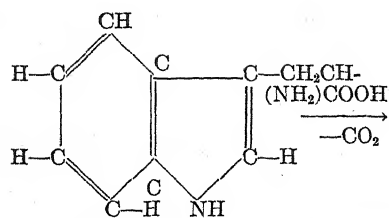
Before discussing the defense mechanism of the body, we will consider the manner in which toxic chemical substances are continually formed in the intestine. The unabsorbed food and even the intestinal juices are subjected to the action of countless bacteria. Although the hydrochloric acid of the stomach acts as a mild disinfectant in normal cases, still there is a large enough bacterial flora in the intestinal tract to bring about some putrefaction of the protein material. Intestinal bacteria are found in ever-increasing amounts from the pylorus to the anus. It has been estimated that the number of bacteria excreted per day vary between thirty and one hundred and thirty trillion. It has been claimed by some investigators that 20-30 per cent of the dry feces is made up of dead bacteria. It is entirely erroneous to maintain that the amount of putrefaction is proportional to the amount of

protein eaten or even to the amount of protein unabsorbed from the intestine. In most cases the amount of putrefaction is proportional to the length of time a given quantity of unabsorbed protein remains in the intestine. With each extension of time, there is a rapid increase in the number of bacteria; and this may account for the usual symptoms following a period of constipation, such as general irritability, sleeplessness, loss of appetite, with perhaps nausea and vomiting or a period of melancholia and general mental depression.

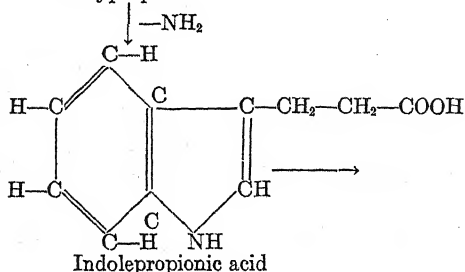
It seems most probable that the bacteria first decompose the protein molecule into amino acids by a process similar to that employed by the proteolytic enzymes in normal digestion. The amino acids are then further decomposed, not according to the normal method of deamination and oxidation, but instead lose CO_2 from the carboxyl group, with the formation of the corresponding amine; or else with a loss of the amino group and the conversion into the corresponding fatty acid. This acid may then be shortened, whether it be a simple aliphatic acid or the side-chain of an aromatic acid. This is brought about by the further action of the bacteria.

The following are a few examples of the action of bacteria on amino acids showing the formation of some of the more common putrefaction products:

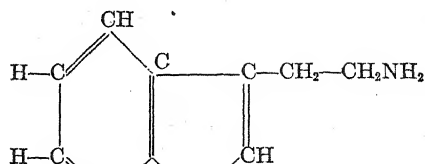




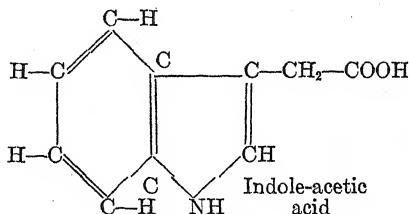
Tryptophan



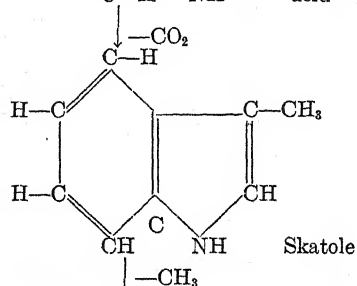
Indolepropionic acid



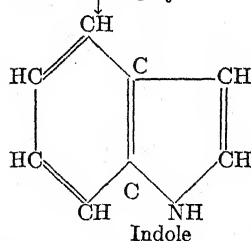
Indole-ethylamine



Indole-acetic acid



Skatole



Indole

DETOXICATION

Under this term we shall include all those chemical changes taking place in the animal organism which have a tendency to render less harmful any poisonous compound or to aid in the rapidity of their elimination. In this chapter only a general outline of the subject of detoxication will be considered. A detailed account, as well as references to the literature, may be found elsewhere.^{1, 2}

When poisons or foreign chemical substances are introduced into the animal body by way of the gastro-intestinal tract, or directly into the tissues by injection, the defense mechanism of the body has a definite method of attack. The first attempt is at complete destruction of the compound through *oxidation*. If the compound resists oxidation or if it can be only partly oxidized, the body then attempts

a *reduction* of the substance. If these processes fail the organism then resorts to a compromise; that is, *conjugation* of the toxic substance with another molecule or substance, which renders the invading molecule less toxic and, as a rule, more soluble.

There have appeared at times a few reactions which might point to the fact that conjugation precedes oxidation. It was suggested by Sherwin several years ago that the formation of ethereal sulfates might well be due to the conjugation of some phenolic substance with cysteine, which was then later oxidized to an ethereal sulfate. Quick recently has suggested that perhaps the chief reason for the formation of glucuronic acid combinations is to increase the ease of oxidation, and that glucuronic acid conjugates are even employed in the burning of the fatty acids. It also must be remembered that the same substance is at times detoxicated in quite a different manner by different species of animals.

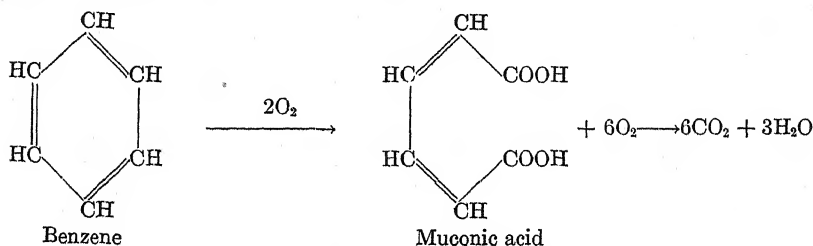
Knoop noticed that whenever a derivative of an aromatic fatty acid was fed to an animal, it appeared in the urine either as a benzoic acid or a phenylacetic acid combination with glycine; that is, either as hippuric acid or as phenaceturic acid.³ A detailed study of this situation showed that benzoic acid "formers" were invariably those containing an odd number of carbon atoms in the chain (such as phenylpropionic or phenylvaleric acid), while those containing an even number of carbon atoms in the side chain (like phenylbutyric and phenylcaproic) always produced phenylacetic acid. No acid with an odd number of carbon atoms in the side chain ever furnished an intermediary product of metabolism with an even number of carbons. Accordingly, he concluded that these compounds must be shortened by the splitting off of two carbon atoms at a time; that is to say, the β -carbon was in each case the vulnerable link in the chain.

This view of Knoop's was supported by the work of Dakin⁴ who detected β -phenyl- β -hydroxypropionic acid in the urine after feeding phenylpropionic acid. This would indicate that the method of oxidizing the foreign organic compound is probably the same as that employed in burning the normal fatty acids. The body seems powerless in its attempts to oxidize a benzene ring when only one or two carbon atoms are attached. Only when there is a 3-carbon atom chain with an amino, a hydroxy or a keto group occupying the α -carbon atom does the body seem able to completely oxidize the benzene ring. This apparently holds also for longer chain aromatic acids, provided the carbon atoms are of an uneven number so that they may be oxidized to a 3-carbon atom chain, forming either α -hydroxy, α -amino-, or α -keto-propionic acid.

It is interesting to note that when two side-chains are attached to the benzene ring only one is oxidized, and this takes place as if only one chain were present. The dicarboxylic acids are oxidized with difficulty. The aliphatic members of this series have been found to be nephropathic and it is questionable to just what extent they are oxidized. It has recently been shown that phenylglutamic acid when in-

jected into dogs and rabbits is oxidized both at the α - as well as the β -position.⁵ Unsaturated acids are oxidized in much the same manner as the corresponding saturated compounds. For instance, cinnamic acid is oxidized to benzoic acid, the phenylisocrotonic acid to phenylacetic acid.

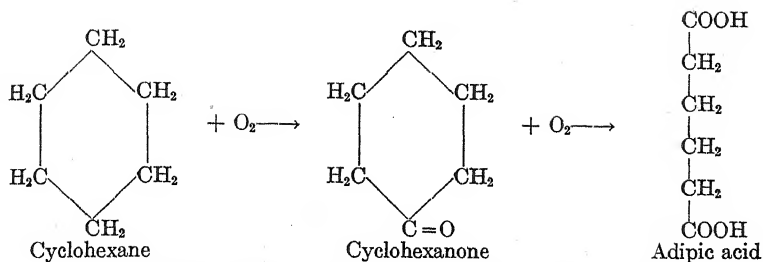
Considerable speculation has been indulged in regarding the oxidation of the benzene ring. Jaffe discovered an unsaturated fatty acid in the urine of animals after feeding benzene and was able to isolate muconic acid.⁶ After injecting muconic acid into rabbits in amounts as large as 2 Gm., he was able to recover only 1 per cent of the substance unchanged. From this work it might be concluded that about 30 per cent of the benzene injected is split into muconic acid; but the latter being easily oxidizable is further burned to water and carbon dioxide, leaving only a trace of the intermediary product of metabolism for excretion:



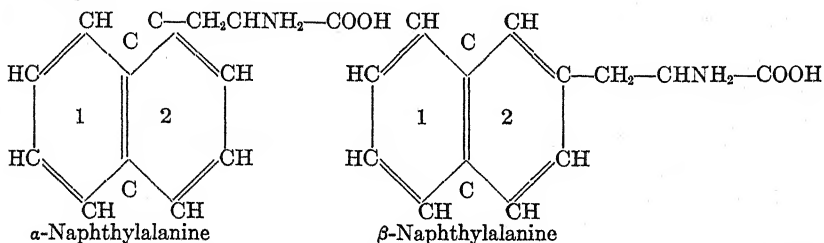
Both the saturated and unsaturated acids behave similarly in the organism in so far as oxidation is concerned. Adipic acid is oxidized with great difficulty; it seems, therefore, improbable that the corresponding unsaturated acid (muconic acid) should be so readily oxidized. Mori repeated the work of Jaffe and was unable to substantiate his claims.⁷ Mori found, on the contrary, that after injecting 0.8 Gm. muconic acid into a rabbit, 73 per cent of the substance was excreted unchanged; and adipic acid injected in the same quantities was eliminated as such to the amount of 61 per cent. After administering adipic acid there was always an increase in oxalic acid excretion as a result of the β -oxidation of some of the adipic acid; but not even this evidence of oxidation was noted in the case of muconic acid. So far we have no *proof* of the destruction of the benzene ring in the body.

As a rule, the aromatic alcohols, like the aliphatic alcohols, are oxidized to the corresponding acids; for example, phenylethyl alcohol is oxidized to phenylacetic acid; *o*-nitrobenzaldehyde and *o*-aminobenzaldehyde are oxidized to the corresponding benzoic acids.⁸ Aromatic hydrocarbons are oxidized to the corresponding acids; for example, *m*-xylene to *m*-toluic acid; *p*-cymene to cumic acid; mesitylene to mesitylenic acid; while pseudocymene is oxidized to xylic acid.

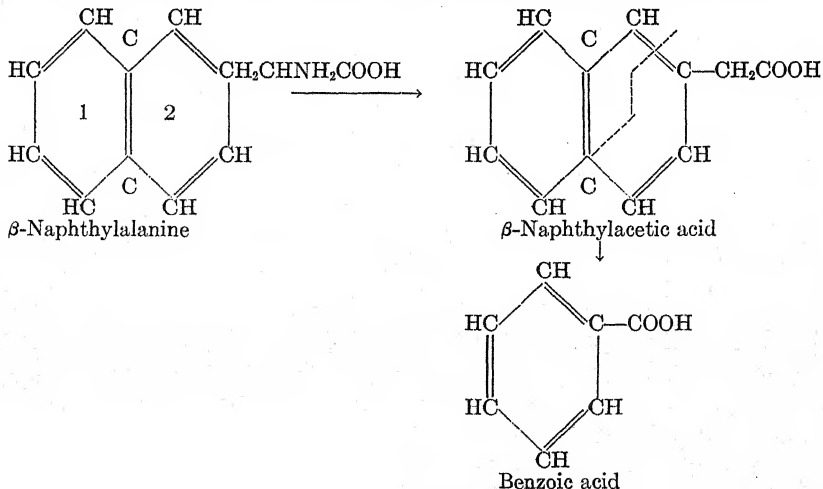
There are also some interesting examples of ring oxidations. Cyclohexane, for example, is oxidized in a large measure to cyclohexanone and small amounts of this compound are further split to adipic acid.



The naphthalene structure is also partially destroyed in the animal body, as was shown by the interesting experiments of Kikkaji.⁹ He wished to compare the fate in the body of a double ring structure containing an α -aminopropionic acid side-chain with a similar benzene ring compound (phenylalanine). He prepared two different compounds: α -Naphthylalanine and β -naphthylalanine and then fed them to dogs:



After feeding the α -compound, he was able to isolate from the urine a substance of uncertain composition which apparently had both rings intact. After feeding the β -compound, however, he found that the side-chain had first been deaminized and simultaneously shortened to two carbon atoms; and then that a second reaction had split ring 2 with the subsequent formation of benzoic acid, which was excreted as hippuric acid. The reaction may be pictured below as follows:



He concluded from this that α -amino-acids are shortened by one carbon atom at a time, and that the ring-splitting occurs between carbon atom 1 and 2 instead of between 3 and 4. If this had not been the case, the resulting compound would have been phenylbutyric acid, which, in turn, would have been oxidized to phenylacetic acid and would have been found in the urine as phenaceturic acid.

Quinaldine fed to rabbits is oxidized to α -picoline, forming an acid resembling picolinic acid. *o*-Nitrocinnamic acid when fed to dogs is partly oxidized to *o*-nitrobenzoic acid, but there seems to be no evidence of quinoline ring formation. Quinoline fed to dog is recovered as methyl quinolinium hydroxide. When fed to rabbits, it is partly oxidized yielding a product which appears in the urine as an ethereal sulfate. Methyl quinolinium hydroxide when fed to dogs, rabbits and chickens is found to be less toxic than quinoline and is excreted unchanged in the urine.³³

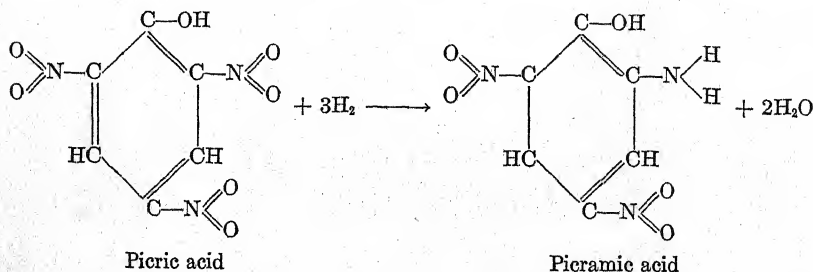
Benzylamine is excreted as hippuric acid after feeding to dogs.

Investigation of the fate of aromatic cyanides showed that *p*-nitro-, *p*-chloro-, and 2,4-dinitrophenyl cyanides, as well as the *p*-chloro-, *o*-nitro-, and *p*-nitrobenzyl cyanides were to a slight extent hydrolyzed into the corresponding acids. There was no splitting off of the cyanide group but there was a decided increase in the output of ethereal sulfates.¹⁰ Triphenylmethane, triphenylcarbinol and triphenylacetic acid were excreted largely unchanged by animals, while diphenylacetic acid was excreted by humans as well as by animals in combination with glucuronic acid.¹¹

REDUCTION

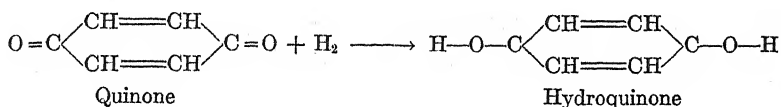
This reaction is much less common than oxidation; however, the tissues have the power to bring about some very remarkable reductions. If we may include the reactions in the intestine under this category, we find that biliverdin and bilirubin of the bile are here reduced to urobilin and still further to the urobilinogen. Ferric salts are reduced to the ferrous condition and bismuth salts are reduced to the lower oxides. Sulfur and even sulfates are reduced to hydrogen sulfide.

One of the most interesting examples is the reduction of picric acid to picramic acid as shown below:

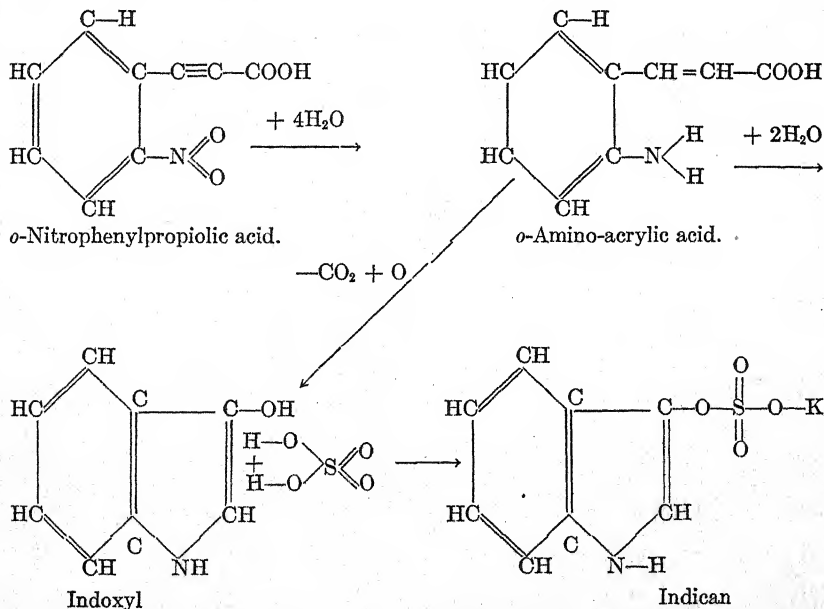


Other well-known examples of reduction are the transformation of *m*-nitrobenzoic acid into *m*-aminobenzoic acid. The *p*-nitrobenzoic acid undergoes the same reaction. Oxidation and reduction may go on simultaneously as is shown by the fact that when *p*-nitrobenzaldehyde is fed to rabbits it is excreted in the urine as *p*-aminobenzoic acid. Chloral is reduced to trichlorethyl alcohol: $\text{CCl}_3\cdot\text{CHO} \rightarrow \text{CCl}_3\cdot\text{CH}_2\text{OH}$; and it is not uncommon to find a carbonyl group reduced to a secondary alcohol group, as such a reaction often precedes a conjugation with glucuronic acid.

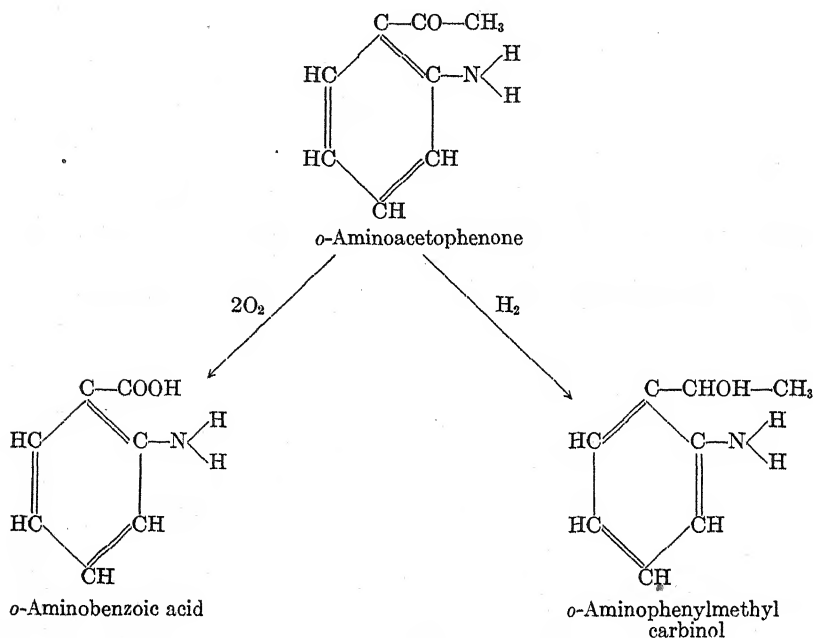
Quinone is reduced to hydroquinone



A most interesting case of reduction is that observed by Hoppe-Seyler.¹² After feeding *o*-nitrophenylpropionic acid to rabbits, he found a substance in the urine identical with urinary indican. The reaction is apparently that represented below. There seems to be a reduction of the nitro to an amino group; also a reduction in the side-chain with the formation of *o*-amino-acrylic acid; then a ring closing with the formation of an indole ring. This compound, through loss of carbon dioxide, is converted into indole itself, which, like ingested indole, is transformed into indoxyl and eliminated as indoxyl potassium sulfate (indican).



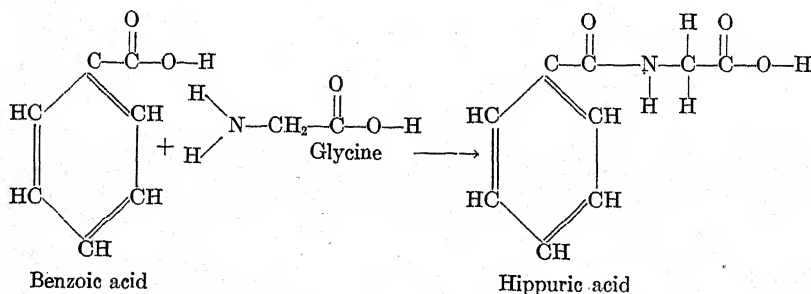
An interesting example of simultaneous oxidation and reduction has been described by Inagacki¹⁵ who found that after injecting *o*-aminoacetophenone subcutaneously into rabbits, *o*-aminophenylmethyl carbinol and *o*-aminobenzoic acid are produced.



CONJUGATION

The synthetic reactions which follow the failure of the body to destroy a compound through oxidation or reduction are limited in number. The substances used by the animal body are glycine, glutamine, cysteine, ornithine, sulfuric acid, acetic acid, glucuronic acid and the methyl group. These will now be considered.

Glycine.—Hippuric acid has been known for more than one hundred years as a conjugation product of benzoic acid with glycine:



It has since been found that glycine is used for the detoxication of benzoic acid not only by the horse but by every vertebrate (except the fowl) so far encountered. Most of the derivatives of benzoic acid (such as the halogen compounds as well as the nitro and amino substitution products) are to some extent combined with glycine. Naphthoic acid also combines with glycine. In fact, the glycine conjugation products run into the hundreds and include not only aromatic acids but pyromucic acid, nicotinic acid, etc.

Glycine is not an essential amino acid. It is not stored in the body. It is furnished for detoxication reactions in much greater amounts than is supplied by the food. Therefore one is forced to conclude that glycine is in some way synthesized by the body. McCollum and Hoagland¹³ showed that when a pig was reduced to a condition of minimal nitrogen metabolism by a carbohydrate diet and at the same time was fed increasingly large doses of benzoic acid, as long as the dose did not exceed 0.2 Gm. per kilo, there was no increase in protein catabolism as evidenced by the constancy in the daily total nitrogen output. Furthermore, there was little alteration in the various nitrogen constituents of the urine with the exception of urea, which could be reduced from 56 per cent of the total nitrogen to 19 per cent. When larger doses of benzoic acid were fed, there was a decided increase in the endogenous catabolism without a corresponding increase in the amount of glyccoll formation. These experiments were corroborated by Lewis and by Shiple and Sherwin on human beings.

From these experiments it would seem that the body has the power to synthesize glycine from the nitrogenous substances of the tissue—not from “extra destroyed protein” as suggested by some experimenters, but from waste nitrogen which results from normal endogenous catabolism and which would otherwise have been excreted in the form of urea. This does not necessarily mean that the body takes the urea and remodels it into glycine; for in all probability the benzoic acid is conjugated with a more complex molecule resulting from endogenous catabolism, which is then converted into hippuric acid.

Had this conjugation with benzoic acid not occurred, the nitrogen used to form glycine would have run the normal course of endogenous catabolism and appeared in the urine as urea. If urea is added to the diet of an animal maintained on a diet as described above, there is no increase in the amount of glycine formed.

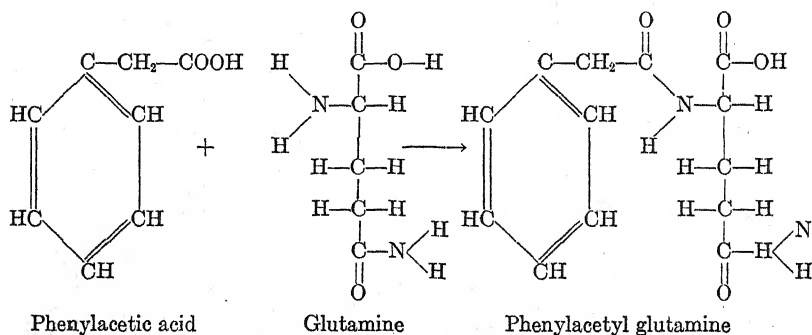
Griffith and Lewis believe that the rate of hippuric acid formation is directly proportional to the amount of glycine available.¹⁴ They administered glycine simultaneously with the feeding of benzoic acid to rabbits. They do not think this was due to the specific stimulation caused by glycine, since alanine had no such marked effect. Simultaneous administrations of alanine, cystine, leucine, norleucine, isovaline, aspartic acid, glycollic acid, glycol aldehyde, glucose or urea with sodium benzoate cause no increase in the rate of hippuric acid

secretion; from which the experimenters conclude that none of these substances are readily available precursors of glycine.

So far as we know, glycine is used only in the detoxication of acids, of which hippuric acid is the best known example. It must also be remembered that the quantity of glycine available is not unlimited. When the glycine is exhausted the body resorts to the detoxication of the foreign organic acid by means of glucuronic acid.

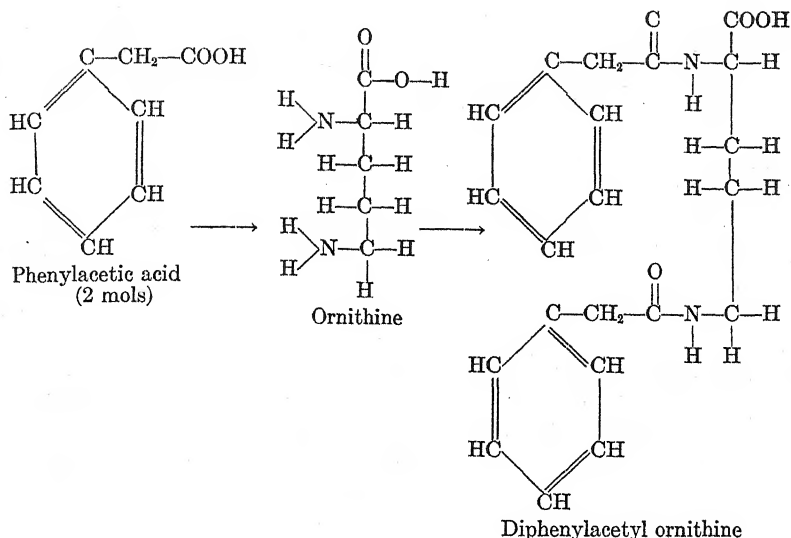
Glutamine.—Glutamine (the amide of glutamic acid) is undoubtedly the component of the protein molecule rather than glutamic acid; for the analysis of Abderhalden and Osborne has shown that the amount of ammonia formed during protein hydrolysis is always proportional to the amount of glutamic acid present.

Phenylacetic acid when fed to animals is combined with glycine to form phenaceturic acid. When ingested by human beings it combines with glutamine to form phenylacetyl glutamine.¹⁶



Man alone seems to have the power to form this compound. More than thirty different derivatives of phenylacetic acid have been ingested by human beings in order to discover, if possible, another glutamine conjugation product. Among these are the various halogen derivatives, the nitro and amino compounds, as well as the hydroxy and methoxy compounds; but in no case was a glutamine conjugate discovered. In addition to these, *o*-brom, *o*-aminophenylacetic acid as well as mandelic acid were tried. Glutamine is a nonessential amino acid, and like glycine it can be synthesized at the expense of the waste nitrogen which would otherwise have been excreted as urea.¹⁷ A small amount of phenylacetic acid is always combined with glucuronic acid, but this never exceeds 10 per cent regardless of the size of the dose.⁴⁸

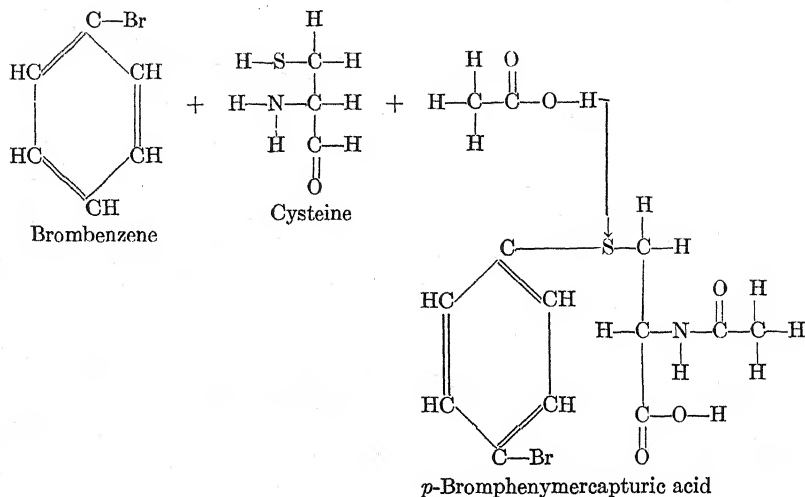
Ornithine.—Ornithine is used only by the fowl as a detoxicating agent. It is employed for the detoxication of organic acids in the same way as glycine and glutamine are used by human beings and animals. The fowl does not seem able to use glycine, glutamine, cysteine, sulfuric or acetic acid in detoxication reactions.



Crowdle and Sherwin provided chickens with an artificial anus, whereby they were able to separate the urine from the feces. They fed them benzoic acid and isolated ornithuric acid from the urine.¹⁸ The ornithine for the detoxication of the benzoic acid was synthesized in spite of the fact that the chickens were on a nonnitrogenous diet. The nitrogen for the synthesis of the ornithine appears to have been taken from the uric acid fraction. The ingestion of arginine increased the output of ornithine, while other amino acids—even histidine—seemed to have little effect. It must be remembered in this connection that uric acid is the chief end-product of nitrogen metabolism in the case of the fowl, comparable to urea in the case of other animals.

It would seem that ornithine, at least, can be synthesized in the body of the chicken. Inasmuch as it is an important constituent of the amino acid arginine, it may help to answer the question, is arginine an essential amino acid—can arginine be synthesized in the organism?

Cysteine.—The fourth amino acid to be used by the body in detoxication is cysteine. It was found both by Jaffe and by Baumann and Preuse that brombenzene, chlorbenzene and iodobenzene, when fed to dogs, are excreted as a sulfur-containing compound known as mercapturic acid. Analysis of the compound showed it to be a halogen derivative of benzene combined with cysteine, which was then acetylated through the amino group of the cysteine:



Inasmuch as the cysteine combines with the benzene ring in the *p*-position it has been taken for granted that the bromobenzene was first oxidized to bromphenol. This does not seem logical, since *p*-, brom-, and *p*-chlorphenol when fed to dogs are excreted as the ethereal sulfate rather than as a mercapturic acid.

Sherwin and coworkers have confirmed the results of earlier investigators who concluded that no mercapturic acid was formed on a low protein or carbohydrate diet.¹⁹ Sherwin *et al.* devised an experiment calculated to test the animal's ability to synthesize cystine. Dogs were maintained on a carbohydrate diet and received bromobenzene along with various forms of sulfur and nitrogen. Although sodium sulfate, potassium sulfocyanide, calcium sulfide, taurine, ethylamino-mercaptan and cystine were fed, mercapturic acid could be isolated from the urine only after cystine feeding in conjunction with the bromobenzene. This naturally led to two conclusions: First, that cystine could not be synthesized from the nitrogen and sulfur compounds which were furnished; and, secondly, that cystine, unlike glycine, glutamine and ornithine, could not be synthesized at the expense of "refuse" nitrogen. This failure of the body to synthesize cystine corroborates the findings of various experimenters who have noted that cystine is a necessary component of the diet; hence an essential amino acid. Mercapturic acids have been reported in the urine of the rabbit after bromobenzene feeding; and in the urine of rats, after feeding iodo-benzene.

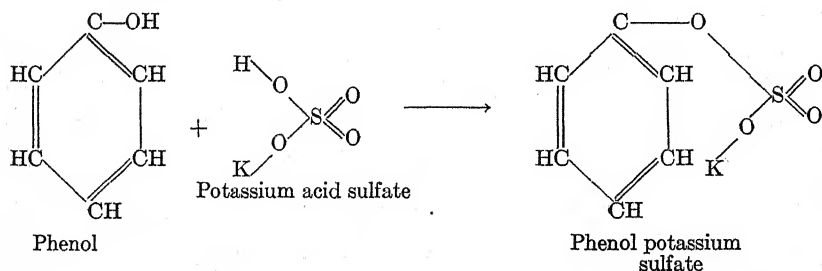
Nishimura reports the isolation of mercapturic acid from the urine of fasting rabbits.²⁰ It seems probable that the cystine used for this synthesis must have been furnished by the body the first day before the fasting had depleted the supply of the body; for in this experiment no daily partition of the urine was made, but the urine for the entire

period was extracted at one time. Abderhalden's experiments on fasting dogs showed that the small amount of available endogenous cystine is immediately used for such body needs as the formation of glutathione, taurine and insulin.²¹ This investigator, however, believes that cystine is formed in tissue catabolism, and that it can be used to detoxicate brombenzene when there is no protein in the diet, provided that the experimental conditions are not extreme.

More recently, White and Lewis have studied the metabolism of brombenzene in dogs on a cystine-deficient protein diet and cystine-rich protein diet and found that mercapturic acid formation (as judged by increase in neutral sulfur) parallels the cystine content of the protein.²² Still more interesting is their simultaneous administration of brombenzene and methionine, to determine whether the latter could take the place of cystine in the formation of mercapturic acid. They found that methionine was apparently as efficient as cystine in protecting the organism against excessive tissue catabolism and the toxic symptoms produced by brombenzene on a low protein diet.

Ethereal Sulfates.—Sulfuric acid is used by the body for the detoxication of aromatic hydroxy compounds, such as phenols and allied substances. Aromatic alcohols, aldehydes and even acids with a hydroxyl group in the nucleus, are often combined with sulfuric acid. In many cases, oxidation of an aromatic compound in the para position causes the formation of an accessible linkage for the acid.

It has been suggested that even the terpenes as well as the purines may combine with sulfuric acid to form a combination which, taken collectively, is called ethereal sulfates. The sulfuric acid combination is usually attached to some alkali metal (such as potassium) and excreted in this form. A good example of an ethereal sulfate is phenol potassium sulfate.



The amount of ethereal sulfate excreted per day varies between 0.1 and 0.3 Gm. It forms about 10–20 per cent of the total sulfate output in twenty-four hours.

The origin of the ethereal sulfate has led to much speculation. The simplest explanation of its formation is to say that it arises directly by a combination with the inorganic sulfates; and this no doubt does occur to a considerable extent. There are cases of phenol

and cresol poisoning where the available supply of sulfuric acid was insufficient to combine with the poison, and the entire output of oxidized sulfur appeared as ethereal sulfate, to the total exclusion of the inorganic portion.

There is another explanation, however. Folin many years ago pointed out that the amount of inorganic sulfate in the urine runs parallel with the urea output and may therefore well be considered as resulting from exogenous metabolism.²³ The excretion of neutral sulfur, however, is much less affected by the amount of protein ingested, and therefore is probably derived from endogenous metabolism, from the breakdown of body tissue and from sulfur existing in the bile. This theory is supported by the fact that when cystine is fed it is chiefly excreted as inorganic sulfate, while injected taurine appears mostly as neutral sulfur.

Shiple and Sherwin postulated the theory that ethereal sulfates might well be due to the oxidation of some sulfur complex such as a mercapturic acid.²⁴ After feeding such compounds, these experimenters found mercapturic acid partially oxidized to ethereal sulfates.²⁵ They also found that inorganic sulfates are ineffective for the detoxication of phenols.

Hele and coworkers published several articles covering their experiments on the same topic.²⁶ Contrary to the findings cited above, these experiments found an increase in the amount of ethereal sulfates after simultaneous feeding of phenolic compounds and sodium sulfate, and found that the increase in ethereal sulfate is at the expense of the inorganic portion.²⁷ After feeding iodophenylmercapturic acid to a pig, they found 83 per cent of the sulfur in the form of neutral sulfur and no increase in ethereal sulfates.

The importance of sulfuric acid as a detoxicating agent has no doubt been greatly exaggerated, due to the fact that it is closely linked, clinically, with putrefactions in the intestine, and that it has been deemed by many to be an index of the amount of putrefaction in the intestine. At most there is only a small amount of sulfuric acid available for this mechanism, since the acid can be replaced by glucuronic acid. It is still a question whether glucuronic acid or the sulfate radical is first employed by the body in emergency cases. In fact, it seems to differ in different species of animals and may even differ with different chemical substances.

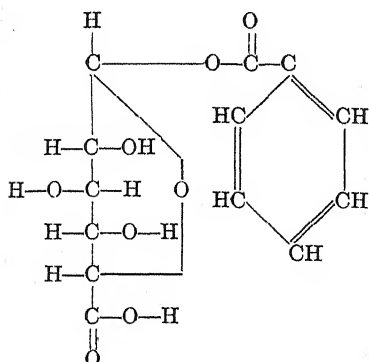
Glucuronic Acid.—Glucuronic acid has been reported in almost an unlimited number of cases. In most instances the compounds fed have contained hydroxy groups or perhaps aldehydes or ketones which, through reduction, have been converted into primary or secondary alcohols. More recently it has been shown that glucuronic acid is quite capable of combining with organic acids, such as benzoic acid and phenylacetic acid, thereby taking the place of glycine.

Unfortunately, many of the studies in glucuronic acid have been confined to a qualitative or "quantitative" test for reducing substances

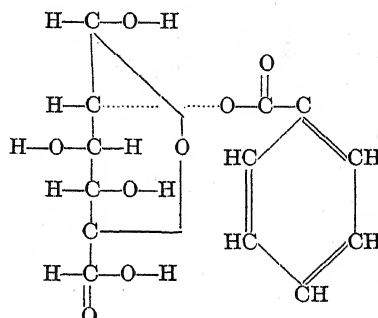
in the urine; and attempts to check many of these results have not proved satisfactory.

Recently, the isolation of pure glucuronic acid compounds from the urine by Quick²⁸ has stimulated research in this direction. The formulas for the glucuronic acid conjugates are particularly interesting since one is an ester type and the other a glucoside binding.

Benzoic acid combines with glucuronic acid to form an ester type of compound. According to Magnus-Levy, it has the structure represented in formula I; according to Quick it has the formula represented by II.

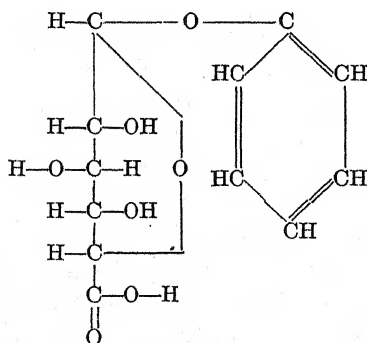


Benzoylglucuronic acid
I



Glucuronic acid monobenzoate
II

Formula III represents the glucoside type of binding which exists between a hydroxy compound such as phenol and glucuronic acid.



Phenolglucuronic acid
III

Recent work has shown that glucuronic acid plays a much greater rôle in detoxication than was formerly thought. In the detoxication of organic acids it takes the place of glycine when the body is unable

to supply a sufficient amount of this material; and it also seemingly has an unlimited capacity for the detoxication of aliphatic and aromatic compounds after the supply of ethereal sulfate has been exhausted.

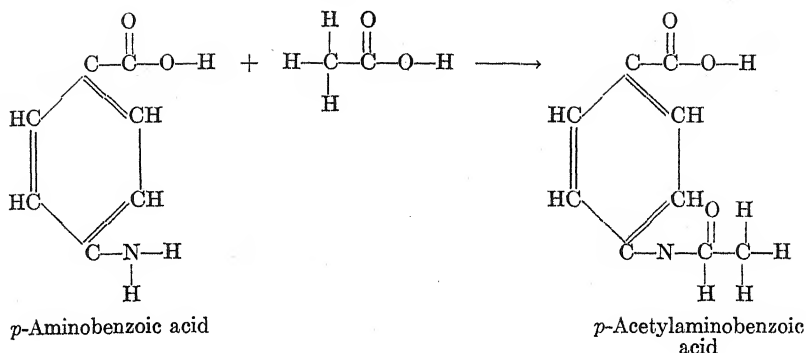
The question which has perplexed investigators for more than fifty years is concerning the origin of glucuronic acid. It has now been generally accepted that this substance arises from glucose; but it still remains to be determined whether the acid is a product of normal glucose metabolism or whether it represents an abnormal pathway in the intermediary metabolism of glucose.

Emil Fischer many years ago suggested that the glucoside type arises from a combination of some substance containing a hydroxyl group with glucose, and that an oxidation then follows which converts glucose into glucuronic acid. This phase of the question has recently been investigated by Hemingway, Pride and Williams, who proved that glucuronic conjugates were formed in the liver.²⁹ They perfused the liver with blood containing β -phenyl-*d*-glucoside and β -bornyl-*d*-glucoside. At no stage after the addition of either glucoside was any increase of glucuronic acid conjugate noted in the urine.

Quick isolated a most unusual compound from the urine after feeding *p*-hydroxybenzoic acid. In this instance one molecule of glucuronic acid combined with the carboxyl group of the acid in ester linkage, while the second molecule was attacked through the *p*-hydroxyl group in glucoside binding. This is the only diglucuronic acid compound so far reported, although it is believed that salicyclic acid may undergo the same transformation.

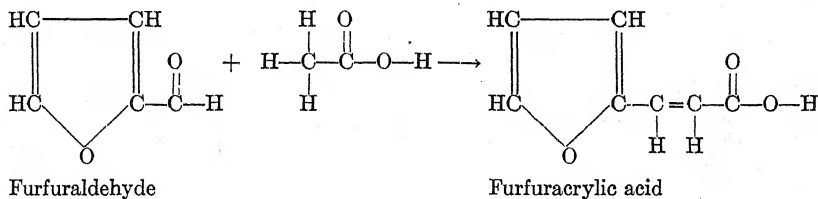
It has previously been shown that the organism has a limited capacity for burning glucuronic acid and that this acid has no power to alleviate insulin convulsions. Quick has studied the relation of this acid to carbohydrate metabolism.³⁰ He has shown that a dog kept on a pure carbohydrate diet can synthesize as much as 5 Gm. of glucuronic acid in twenty-four hours without any increase in nitrogen metabolism. He furthermore demonstrated that when a glucuronogenic drug is fed to a diabetic dog, glucuronic acid is synthesized from the glucogenetic fraction of the protein molecule. Since the administration of insulin greatly increases the output of glucuronic acid, it seems probable that glucose can be utilized for the synthesis of this acid through the action of insulin. Feeding of aceto-acetic acid reduces the output of glucuronic acid. Quick is of the opinion that the initial step in the catabolism of fatty acids may be a conjugation with glucuronic acid.

Acetic Acid.—Acetic acid not infrequently plays an interesting and perhaps important rôle in intermediary metabolism. The usual type of reaction into which it enters is in the acetylation of amino groups:



This example of the acetylation of *p*-aminobenzoic acid is the most familiar one. Another example is the acetylation of the amino group of cysteine in the formation of mercapturic acids. It seems peculiar that acetic acid is never used by the body in the detoxication of hydroxyl groups. In fact, acetylated hydroxy compounds are easily hydrolyzed in the body.

A most unusual case of acetylation is that reported by Jaffe and Cohn.³¹ After feeding furfural to rabbits and dogs, they found that the methyl group of the acetic acid had condensed with the aldehyde of the furfural resulting in the formation of fufuraerylic acid:



This reaction is analogous to the formation of cinnamic acid by the Perkins reaction (from benzaldehyde, acetic anhydride and sodium acetate).

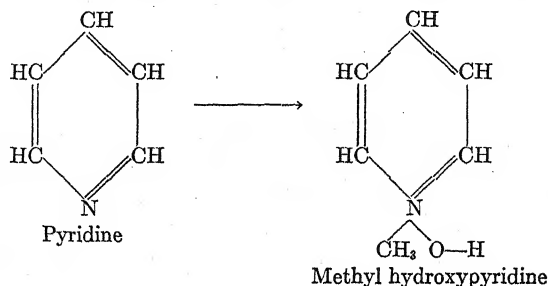
Acetic acid is not stored in the body and only by this means can it be shown that acetic acid is an actual product in intermediary metabolism. Harrow, Mazur and Sherwin³² have studied the acetylation reaction qualitatively and quantitatively. They found that *p*-aminobenzoic acid is acetylated to a greater degree in the body of the rabbit than any other amino compound. Furthermore, they were able to isolate the acetylated compound and weigh it, thereby making the estimation quantitative. Since carbohydrates and possibly fats are the probable sources of acetic acid, it is interesting to note the effect of insulin injections upon the acetylated product produced.

Their results show that the injection of 0.5 unit of insulin per kilo weight raises the acetylation value. Glutathione, on the con-

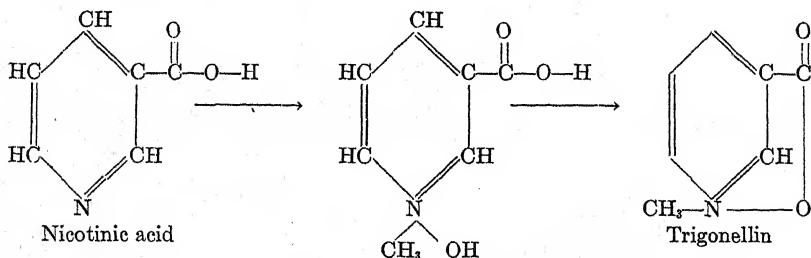
trary, had no effect upon the amount of the acetylated product when injected alone. When reduced glutathione and insulin were injected simultaneously, after feeding *p*-aminobenzoic acid, it was found that the glutathione inhibited the action of the insulin.

Methylation.—The use of the methyl group in plant physiology seems to be one of the commonest reactions. In the animal body, this reaction is not common.

His found that after feeding pyridine to a dog he was able to isolate from the urine a methyl hydroxypyridine compound:



Ackermann later reported the methylation of nicotinic acid in the body of the dog. Here we also have the addition of a methyl and an hydroxyl group to the nitrogen of the pyridine ring, followed by a secondary inner anhydride formation (by the splitting off of a molecule of water):



It is claimed that quinoline injected subcutaneously into dogs is to some extent methylated.³³ Experimenters have fed imidazole, pyridine, quinoline, piperidine and quinaldine to dogs and rabbits in the hope that a quantitative study of the methylation reaction would be possible. Of these substances, only pyridine was methylated in the organism of the dog but no quantitative method could be developed for determining the methylated compound.³⁴

According to Kamei pyridine, quinoline and nicotinic acid when administered to dogs having Eck's fistula, appear (to a small degree) as methylated derivatives in the urine.³⁵ Stuber claims that guanidine acetic acid, injected intravenously into rabbits, is converted into creatine by methylation.³⁶

SITE OF DETOXICATION

It has often been asked where these reactions take place—whether they are limited to one tissue or organ. There is a considerable difference of opinion and a great deal of contradiction in the literature. It may safely be said that the liver is the organ of first importance in the promotion of the detoxication reaction. The kidneys play an important rôle. In many cases it would seem that undue emphasis is placed upon the kidney because it is difficult to say whether a change in the detoxication mechanism caused by a diseased kidney is due to faulty synthetic reactions or to faulty elimination.

Since the early work of Bunge and Schmiedeberg, it has been known that in the dog hippuric acid is synthesized only by the kidney. This resulted in various efforts to utilize the conjugation of benzoic acid as a kidney function test. Quick believes it quite probable that in man, as in the rabbit, the synthesis of hippuric acid also takes place in the liver, and has utilized this fact to determine the amount of liver damage.³⁷

Delpratt and Whipple believe that the liver plays the chief part in the synthesis of hippuric acid, but that other cells can take over the work, if necessary.³⁸ Morgulis, Pratt and Johr state that in nephritis, hippuric acid synthesis is never complete but varies between 53 and 95 per cent of the theoretical amount.³⁹ Snapper finds that men with normal kidneys will excrete 5 Gm. of benzoic acid in twelve hours in the form of hippuric acid.⁴⁰ He believes that the reduced kidney excretion is not due to lack of synthesis by the kidney, but rather to the failure of the kidney to excrete the substance.

Violle believes that the synthesis of hippuric acid is not affected by diseases of the liver, since he finds diminished hippuric acid excretion in all renal disturbances, he also notes that the amount of its excretion runs parallel to the other kidney function tests and to albuminuria.⁴¹

Koyasako removed the thyroid of rabbits then and injected them with benzoic acid. The addition of thyroid powder increased the synthesis. Insulin also increased the output of hippuric acid, while adrenalin seemed to retard the synthesis.⁴²

Hemingway, Pride and Williams have recently conducted a remarkably interesting experiment in which they investigated the seat of glucuronic acid conjugation.²⁹ By means of a "pump-lung preparation," they perfused the isolated kidney alone and then in conjunction with other isolated organs, in order to investigate the reaction of such perfused organs to glucuronogenic and other substances added to the circulating blood. After proving that the kidney is able to excrete conjugated glucuronic acid, they added phenol to the blood and perfused the isolated kidney. Negative results were obtained; that is to say, in the urine formed by this kidney, free phenol was found, but no glucuronic acid conjugate could be shown.

The liver was next perfused with 30 mg. of phenol and placed in

three liters of normal saline. Within four minutes after the addition of each quantity of phenol, using the naphthoresorcinol reaction, marked increases in the glucuronic acid excretion were observed. They also perfused the spleen as well as the tissues of the hind limbs. They came to the conclusion that the liver is the chief if not the only site of glucuronic acid conjugation, and that the formation and elimination of the acid is much more rapid than was previously supposed.

Acetylation takes place largely in the liver and gives a rough quantitative index of the amount of liver damage.

THEORIES CONCERNING DETOXICATION

Several theories have been advanced to explain the detoxicating mechanism. Berczeller suggests an explanation for certain conjugated products. He believes that the compounds which are susceptible of conjugation are those which increase the surface tension of the liquid in which they are dissolved, while the conjugated product has less effect. Sherwin and coworkers repeated these experiments and investigated many more substances, comparing the surface tension of the substance with that of the conjugation product. The results were not at all conclusive.

Abderhalden and Wertheimer are of the opinion that the diet has a great deal to do with the type of conjugation taking place in the organism.⁴³ After injecting brombenzene into rabbits on a diet of oats, they obtained a small amount of mercapturic acid but no trace of the acid when a diet of green vegetables was used; thus indicating that the synthesis is favored by an acid diet and inhibited by an alkaline one.

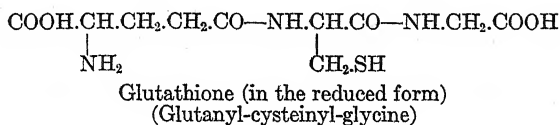
Of the several detoxication processes reported to be dependent on this dietary factor, Griffith showed that hippuric acid synthesis is not dependent upon the diet;⁴⁴ and Braunstein, Parschin and Chalisowa presented striking evidence to show that the extent of oxidation and detoxication of benzene, toluene and phenol is dependent on the diuresis and independent of the acid or basic character of the diet.⁴⁵ Abderhalden and Wertheimer's experiments were repeated in the authors' laboratory and could not be corroborated.

Quick has recently proposed an interesting theory of detoxication.⁴⁶ He believes that the object in detoxication is to form a strong acid out of a slightly acidic compound or to convert an inert compound into an acid. Toluene, for example, is oxidized to benzoic acid. The ionization constant of benzoic acid is 6.5×10^{-5} whereas that of hippuric acid is 2.3×10^{-4} and that of glucuronic acid monobenzoate, 3×10^{-3} . Similarly, phenylacetic acid has an ionization constant of 5.6×10^{-5} and phenaceturic acid, 2×10^{-4} . While menthol is a neutral compound, menthol glucuronic acid has a constant of approximately 1×10^{-3} . In fact, all the glucuronic acids (both of the glucoside and ester type) are relatively strong acids. Phenol, a very weakly acidic compound, is strongly acid when combined with sulfuric acid. *p*-Brombenzene is converted into a fairly strong mercapturic acid; and even

acetylation of *p*-aminobenzoic acid develops a more strongly acid compound. He believes that mandelic acid and *o*-nitrogenzoic acid are excreted unchanged because they are already strong acids. *p*-Hydroxybenzoic acid seems to be an exception to this rule, as it appears in the urine unchanged. It is suggested that in this instance this relatively weak acid passed through the body as a glucuronic acid conjugate and is later broken off.

GLUTATHIONE AND DETOXICATION

A glance at the formula for glutathione shows this substance to be the ideal detoxicating agent:



Theoretically, it is quite easy to picture any one of a number of reactions taking place, whereby cystine, glycine, glutamine, or even sulfuric acid, acetic acid, ornithine and glucuronic acid could be derived from this tripeptide. In fact, it is easy to believe that phenylacetic acid, for example, might be linked with the free amino group of the glutamic acid in an attempt to oxidize the phenylacetic acid. When this fails, a part of the glutathione molecule is then oxidized to glutamine. Or in the formation of mercapturic acids, the brombenzene might combine with the free —SH group of the glutathione and in this way (through disintegration of the glutathione) obtain its cysteine.

For the past ten years, many theories have been advanced suggesting that glutathione might be involved in detoxication reactions, but as yet little experimental work has been done. Waelsch ingested phenylacetic acid as the sodium salt to ascertain what effect the detoxication of this acid with glutamine might have on the glutathione content of the blood. He found a reduction of about 20–25 per cent in the glutathione content of the blood one and a half hours after taking the acid, and he believes this reduction in glutathione content indicative of its use in the detoxication of the phenylacetic acid.⁴⁷ Harrow, Mazur and Sherwin³² studied the effect of glutathione on the acetylation of *p*-aminobenzoic acid. It was found that glutathione (5 mg. reduced glutathione per kilo of body weight) injected at the time of feeding *p*-aminobenzoic acid (1 Gm. per day) had no effect on the amount of the acid acetylated. Insulin increased the amount of acetylation while insulin and glutathione injected at the same time had no effect; showing that glutathione has a neutralizing effect on insulin.

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REFERENCES

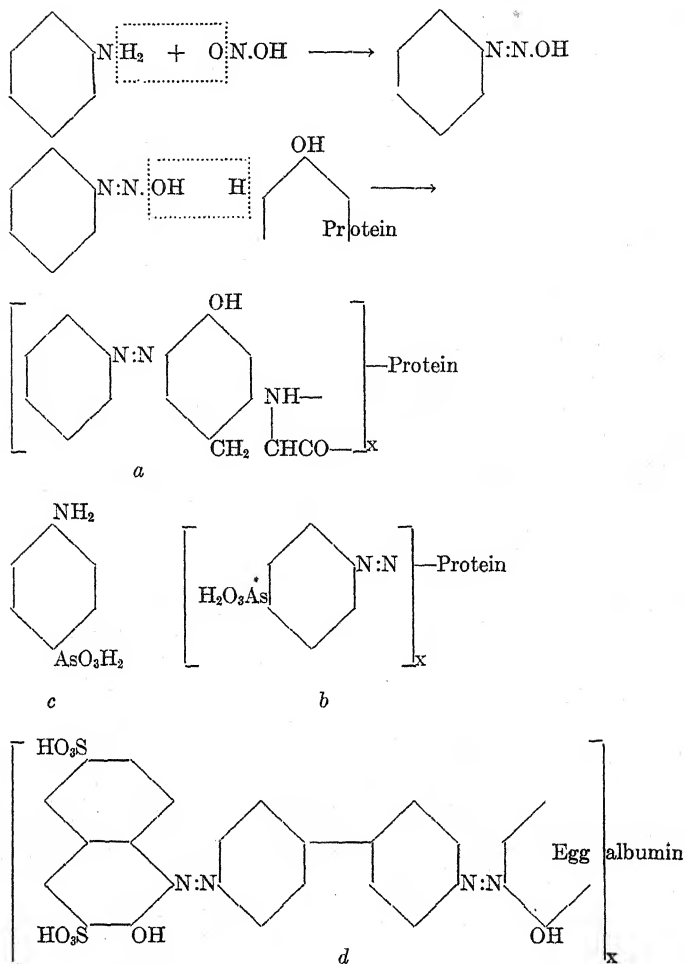
1. Sherwin, C. P.: *Physiol. Rev.*, **2**, 238 (1932); **2**, 238 (1922).
2. Ambrose, A. M., and Sherwin, C. P.: *Annual Rev. Biochem.*, **2**, 377 (1933).
3. Knoop, E.: *Z. physiol. Chem.*, **67**, 489 (1910).
4. Dakin, H. D.: *J. Biol. Chem.*, **6**, 203 (1909).
5. von Beznak, A.: *Biochem. Z.*, **205**, 420 (1929).
6. Jaffe, M.: *Z. physiol. Chem.*, **62**, 53 (1909).
7. Mori, Y.: *J. Biol. Chem.*, **35**, 341 (1918).
8. Hosoda, T.: *J. Biochem. (Japan)*, **6**, 171 (1926).
9. Kikkaji, T.: *Biochem. Z.*, **35**, 57 (1911).
10. Adeline, M., Cerecedo, L., and Sherwin, C. P.: *J. Biol. Chem.*, **70**, 461 (1926).
11. Miriam, S. R., Wolf, J. T., and Sherwin, C. P.: *J. Biol. Chem.*, **71**, 249 (1927).
12. Hoppe-Seyler, G.: *Z. physiol. Chem.*, **7**, 178 (1882).
13. McCollum, E. V., and Hoagland, D.: *J. Biol. Chem.*, **16**, 311 (1913).
14. Griffith, W. H., and Lewis, H. B.: *J. Biol. Chem.*, **57**, 1 (1923).
15. Inagacki, S.: *Z. physiol. Chem.*, **214**, 25 (1933).
16. Thierfelder, H., and Sherwin, C. P.: *Ber.*, **47**, 2630 (1914).
17. Shiple, G., and Sherwin, C. P.: *J. Am. Chem. Soc.*, **44**, 618 (1922).
18. Crowdle, J., and Sherwin, C. P.: *J. Biol. Chem.*, **55**, 671 (1923).
19. Shiple, G., and Sherwin, C. P.: *J. Biol. Chem.*, **55**, 671 (1923).
20. Nishimura, K.: *Acta Schol. Med. Univ. Imp. Kioto*, **12**, 73 (1929-30).
21. Abderhalden, E., and Wertheimer, E.: *Z. physiol. Chem.*, **198**, 18 (1931).
22. White, A., and Lewis, H. B.: *J. Biol. Chem.*, **98**, 607 (1932).
23. Folin, O.: *Am. J. Physiol.*, **13**, 66 (1905).
24. Shiple, G., and Sherwin, C. P.: *J. Biol. Chem.*, **55**, 671 (1923).
25. Sherwin, C. P., Shiple, G., and Rose, A. R.: *J. Biol. Chem.*, **73**, 607 (1927).
26. Hele, T. S.: *Biochem. J.*, **18**, 110 (1924).
27. Coombs, H. I., and Hele, T. S.: *Biochem. J.*, **21**, 611 (1927).
28. Quick, A. J.: *J. Biol. Chem.*, **74**, 331 (1927).
29. Hemingway, A., Pride, J., and Williams, R. T.: *Biochem. J.*, **28**, 136 (1934).
30. Quick, A. J.: *J. Biol. Chem.*, **70**, 397 (1926).
31. Jaffe, M., and Cohn, R.: *Ber.*, **20**, 2311 (1887).
32. Harrow, B., Mazur, A., and Sherwin, C. P.: *Proc. Soc. Exptl. Biol. Med.*, **30**, 1143 (1933); *J. Biol. Chem.*, **102**, 35 (1933); *J. Biol. Chem.*, **105**, No. 2, May (1934).
33. Tamura, S.: *Acta Schol. Med. Univ. Imp. Kioto*, **6**, 449 (1924).
34. Novello, N. J., Harrow, B., and Sherwin, C. P.: *J. Biol. Chem.*, **67**, 54 (1926).
35. Kamei, T.: *J. Biochem. (Japan)*, **7**, 197 (1927).
36. Stuber, B.: *Klin. Wochschr.*, **2**, 931 (1923).
37. Quick, A. J.: *Am. J. Med. Sci.*, **185**, 630 (1934).
38. Delprat, G. W., and Whipple, G. H.: *J. Biol. Chem.*, **49**, 229 (1921).
39. Morgulis, S., Pratt, G. P., and Johr, H. M.: *Arch. Internal Med.*, **31**, 116 (1923).
40. Snapper, I.: *Klin. Wochschr.*, **3**, 55 (1924).
41. Violle, P. L.: *Compt. rend. soc. biol.*, **84**, 194 (1921).
42. Koyasako, T.: *Folia Endocrinologia Japonica*, **6**, 110 (1931).
43. Abderhalden, E., and Wertheimer, E.: *Arch. ges. Physiol.*, **207**, 215 (1925).
44. Griffith, W. H.: *J. Biol. Chem.*, **64**, 401 (1925).
45. Braunstein, A.: Parschin, A., and Chalisowa, O.: *Biochem. Z.*, **235**, 311 (1931).
46. Quick, A. J.: *J. Biol. Chem.*, **97**, 403 (1932).
47. Waelsch, H., and Weinberger, E.: *Arch. exp. Path. Pharmacol.*, **156**, 370 (1930).
48. Ambrose, A. M., Power, F. W., and Sherwin, C. P.: *J. Biol. Chem.*, **101**, 699, (1933).

CHAPTER XIV

IMMUNOCHEMISTRY

In recent years chemistry has done much to clarify and systematize the knowledge of the complex processes resulting in resistance or immunity to disease. Wells, in his "Chemical Aspects of Immunity"¹ has so thoroughly reviewed the subject that the writer will attempt chiefly to relate certain advances made since the second edition of Professor Wells' book was written, drawing only occasionally on the older material. It is proposed first to recount some of the chemical studies which have led to a clearer understanding of the concept *antigen*, then to develop similarly the present conception of *antibody*, and finally to discuss such chemical knowledge as is available of antigen-antibody interactions.

The Antigen.—Until very recently it seemed certain that only intact protein or very slightly degraded protein could function fully as an antigen and directly stimulate the production of antibodies in animals. However, there is now some evidence that undegraded specific polysaccharides may stimulate the formation of antibodies, as will be discussed later. It is clear, however, that most antigens are proteins of varying degrees of complexity. Landsteiner, to whose studies we are indebted for much of our knowledge of the chemical basis of serological specificity, has introduced the useful term *hapten* for that portion of a complex antigen which determines the serological specificity. The hapten fragment of a complete antigen, as the determinant of specificity, is capable of reacting with antibodies produced by the whole antigen, but by itself rarely stimulates antibody production. Landsteiner has shown that in the complex azoproteins, it is the diazotized aromatic amine which determines the specificity of the resulting compound antigen and may be termed the *hapten*; for whether the protein be derived from the horse or the chicken, the specificity of the new azoprotein is the same. Thus aniline azo horse serum (see the following formula *a*)* stimulates the production of antibodies in the rabbit which react with aniline azo chicken serum, although native horse serum and chicken serum are each species specific and the antisera to them do not cross-react. Since the hapten introduced enters and modifies the tyrosine groupings and perhaps other cyclic groups of the protein, it would appear that these amino acids—chiefly tyrosine and histidine—play an important rôle in the specificity of antigens, and when altered by the introduction of other substances, permit the establishment of a new specificity characteristic of the entering group, or hapten. Alteration of other acidic or basic groups by acylation or methylation also changes the specificity, so that the tyrosine and histidine groups are not necessarily the only



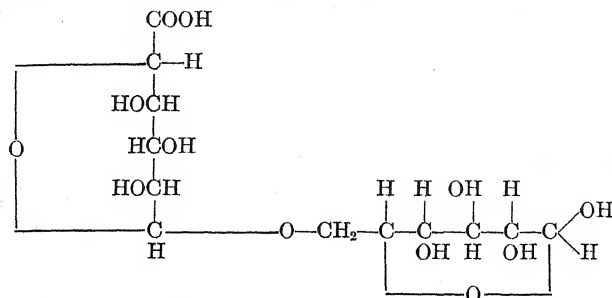
groups concerned. This has again been demonstrated by the addition of phenylisocyanate to protein.²

The question as to what determines the specificity of a hapten has been considered³ and evidence presented to show that it is the "field of force" surrounding a group which is responsible, since groupings with similar force fields are difficult to differentiate immunologically.

Immunologically Reactive Polysaccharides.—In the last ten years it has been found that the proteins are not the only class of naturally occurring substances possessing immunological reactivity. In 1917 it was found⁴ that culture filtrates of virulent pneumococci contained a "soluble specific substance" which was rigorously type specific, giving a precipitate with antiserum to the homologous pneumococcus

* Reprinted from *Medicine*, 12, 279 (1933); *Harvey Lectures*, 28, 184 (1933).

type. Similar reactive products were discovered in extracts of various micro-organisms and called "residue antigens."⁵



ALDOBIONIC ACID FROM TYPE III PNEUMOCOCCUS SPECIFIC POLYSACCHARIDE
Position of attachment of glucuronic acid to glucose is unknown.

A study of the "soluble specific substances" of type II and type III pneumococcus showed that each was a chemically distinct nitrogen-free polysaccharide⁶ and thus established the participation of the sugars in immune reactions and in the determination of bacterial specificity.⁷ Indeed, when one considers the enormous number of polysaccharides possible on account of the many known sugars and the multiplicity of arrangements and isomeric forms in which they may be built up into polysaccharides—a multiplicity and variety approached only by the proteins—it does not appear strange that the polymeric sugars should often have immunological properties. Subsequent work showed the presence of galacturonic acid and a nitrogenous component in the type I substance⁸ and demonstrated that the type III specific sugar was built up of aldobionic acid units (see the formula above)⁹—the first discovery of a type of sugar acid which has since been found in other bacterial polysaccharides and even widely distributed in nature in the plant gums, such as gum arabic, and in the hemicelluloses.

In the following table is given a summary of the properties of the

COMPARISON OF THE TYPE-SPECIFIC POLYSACCHARIDES OF PNEUMOCOCCUS

| Polysaccharide. | [α] _D | Acid equivalent. | Total nitrogen per cent. | Amino nitrogen per cent. | Acetyl nitrogen per cent. | Hydrolysis products. | |
|-----------------|------------------|------------------|--------------------------|--------------------------|---------------------------|----------------------------------|---|
| | | | | | | Calculated as glucose, per cent. | |
| Type I*.... | +300° | 310 | 5.0 | 2.5 | 0 | 28 | Galacturonic acid. (Nitrogenous substance). |
| Type II..... | + 74° | 1250 | 0.0 | | | 70 | Glucose. |
| Type III.... | - 33° | 340 | 0.0 | | | 75 | Aldobionic acid, glucose. |
| Type IV†.... | + 30° | 1550 | 5.5 | 0.1 | 5.8 | 71 | Acetic acid. (Amino sugar derivative). |

* The type I substance as originally isolated has now been shown to be a degradation product lacking acetyl groups attached to oxygen (10).

† Heidelberger, M., and Kendall, F. E.: *J. Exp. Med.*, 53, 625 (1931).

specific polysaccharides of types I, II, III and IV pneumococcus. It would now seem that nearly every species of bacterium is possessed of one or more specifically reactive polysaccharides, and although the connection of these sugars with microbial structure, virulence, and immunological reactions is not always as clear as in the case of *Pneumococcus*,¹¹ they play a definite and often determining rôle in bacterial specificity. While the specific carbohydrates may be classed as haptens, they represent a distinctive group which not only combines with antibody but precipitates it as well. This property may be a function of their molecular weight, which has been shown to be low in comparison with the common proteins¹² but high enough to be in close agreement with values only recently established by Haworth for glycogen and starch, namely 2400 to 6000.¹³ It has also been found that partial hydrolysis products of specific polysaccharides of as low molecular weight as 500 still retain the power to precipitate antibody elaborated by the horse, but do not precipitate antibody formed in the rabbit.¹⁴ Azo dyes coupled with resorcinol, with about the same molecular weight, may also react as precipitating haptens.¹⁵

Carbohydrate Proteins.—A brilliant development of the work on specific polysaccharides has been the synthesis of carbohydrate proteins by diazotization and coupling of a suitable sugar derivative with protein,¹⁶ whereby it was shown that the rotation of a single carbon atom through an angle of 180 degrees was reflected in a change in immunological specificity.¹⁷ The specific polysaccharide of type III pneumococcus was also coupled to horse serum globulin and it was found that the antiserum produced by injecting the product into rabbits agglutinated the type III pneumococcus and even protected mice against many lethal doses of the virulent micro-organism.¹⁸ Mention should also be made of the discovery of an enzyme which strips the type III pneumococcus of its specific carbohydrate and its virulence.¹⁹

There is evidence that the specific carbohydrates may occasionally show limited antigenic properties. Thus the unhydrolyzed acetyl polysaccharide of type I pneumococcus, when injected into mice in minute amounts, confers active immunity against virulent type I pneumococci, but this property is lost on removal of the acetyl groups.^{10A} Antibodies have been reported as following the intradermal injection of pneumococcus specific carbohydrates,²⁰ and it is also claimed that adsorption on inert carriers may render these substances antigenic.²¹

Lipoids.—Another group of natural substances which may perhaps be classed as haptens are the lipoids, although it is beginning to appear as if the specificity formerly attributed to supposed lipoids, such as the group of Forssman antigens and blood group specific substances, may be due to polysaccharides and proteins, respectively. In this connection it is interesting to recall the intimate relation between carbohydrate and lipid in the purified horse-kidney Forssman antigen,²² and the polysaccharides encountered among the lipid fractions of the tubercle bacillus.²³ While there is some evidence that highly specific

antibodies may be developed in a very small percentage of animals following the injection of chemically pure members of the cholesterol group in admixture with pig serum, there is also evidence for and against the forcing of pure lecithin into antigenic combination. Even the chemistry of the "antigen" used in the Wassermann reaction has not been elucidated. We must, therefore, leave the subject of immunological properties of lipoids in its present equivocal state.²⁴

"Bacteriophage."—The chemical nature of "bacteriophage" is still in doubt, so that its classification is uncertain. Its antigenic properties seem established, however.²⁵ The kinetics of its action have been studied²⁶ and a promising method indicated for its purification in one instance.²⁷

The Concept "Antibody."—In his chapter in "A System of Bacteriology in Relation to Medicine,"²⁸ published in 1931, the English immunologist Dean wrote "we have no conclusive evidence that antibodies, in the sense of definite chemical substances, exist." This, I think, is an unnecessarily pessimistic view of existing data, for there is now much evidence that antibodies are actually modified serum globulins. The chief uncertainty is caused by the insistence of certain immunologists that they have obtained protein-free antibody solutions. These workers have forgotten the oft-repeated demonstration that tests for protein with chemical reagents fail at dilutions at which biological reactions such as anaphylaxis and bacterial agglutination readily occur, so that until such experiments result in the isolation of weighable amounts of protein-free antibody they can carry little conviction.

On the other hand, there is much that points toward the actual protein nature of antibodies. It has been shown²⁹ that the protective antibodies in antipneumococcus horse serum are more or less completely precipitated when the serum is added to twenty volumes of slightly acidulated water. About 90 per cent of the serum proteins remain in solution and 60 to 80 per cent of the pneumococcus antibodies are concentrated in the precipitate and may be redissolved in saline and subjected to further purification. By removal of an inactive fraction with acid and treatment with zinc or aluminum salts metal antibody compounds have been recorded which were completely precipitable by the pneumococcus polysaccharide of the homologous type.³⁰ Unfortunately, the globulin solutions left after removal of the zinc or aluminum were specifically precipitable to the extent of only about 80 per cent. Although absolutely pure antibody has not yet been isolated, the goal is very nearly attained, and it should soon be possible to obtain antibody in sufficient quantity to study its differences from normal serum globulin.

In addition to the preparation of nearly pure antibody there is now a mass of quantitative data supporting the protein nature of antibodies. For example, it has been shown³¹ that diphtheria toxin-antitoxin floccules consist mainly of denatured pseudoglobulin—the serum protein fraction with which antitoxin is commonly associated

in the horse—and that the amount precipitated is independent of other serum proteins present or added. Other quantitative evidence will be discussed at a later period.

If antibody is actually modified serum globulin, how is it formed and what is its relation to the antigen? Buchner accounted for the specificity of antibody by the assumption that antigen or antigen fragments actually entered into the antibody complex. Ehrlich abandoned this idea because of the large excess of antibody often produced. Recent evidence against Buchner's hypothesis is, moreover, almost overwhelming. This is especially true of the experiments with atoxyl azoprotein (see formula *b* on p. 390).³² In this arsanilic acid (see formula *c* on p. 390) is the hapten, or specificity determining portion, so that if the specificity of the resulting antibody depended upon the incorporation of specific antigen fragments into the antibody molecule these fragments would necessarily contain arsenic, and it should be possible to detect arsenic in the antibody. However, as much as 30 cc. of antiserum contained no more than the faint traces of arsenic in the same amount of normal serum. Similarly, it was shown that the antibody to a deep red azoprotein, R-salt-azobenzidine-azocrystalline egg albumin (see formula *d* on p. 390), was not red, as was the corresponding hapten, but colorless.³³ Moreover, the quantitative work discussed later shows that the actual amount of antibody formed with the aid of minimal amounts of antigen is so great as practically to preclude the participation of specific antigen fragments in the antibody. How, then, can antibodies be explained?

To the chemist the most reasonable theory is the following,³⁴ although it is as yet without direct supporting evidence. The injected antigen or its partial degradation products may reach the points at which globulin synthesis is taking place in the animal body. In the presence of this foreign protein or its products synthesis is somewhat distorted, and distorted in a way characteristic for the foreign material, so that when the finished globulin encounters the foreign protein once more in the circulation or *in vitro*, interaction is possible.

Now that we have acquired a certain chemical perspective on antigens and antibodies, although many fundamental questions as to both are still unanswered, let us consider the mechanism of their interaction.

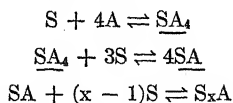
The Mechanism of the Reaction of Antigen and Antibody.—Since most of the substances involved in immune reactions are presumably colloids, the simplest way of disposing of such phenomena as specific precipitation, agglutination, complement fixation or hemolysis, or toxin-antitoxin neutralization is to assume that oppositely charged colloidal particles combine to produce the observed effect. This view was first upheld by Bordet, who later modified it in the sense that the immune reactions represented adsorption phenomena. Ehrlich, on the other hand, insisted that actual chemical combination in definite proportions took place between antigen and antibody, and in this he was supported by Arrhenius and Madsen.

While the colloidal theory offers a possible explanation of the course of events in an immune reaction, it fails entirely to account for the specificity of the reaction. If, for example, one adds to a type I antipneumococcus serum a 1:10,000 solution of the specific polysaccharide of type III pneumococcus, and to a type III antiserum an equal amount of the type I specific polysaccharide, the sera, of course, remain clear. Both mixtures contain antibody globulin, presumably as the ionized sodium chloride compound, and on the alkaline side of the iso-electric point. Charges on the antibody particles are therefore the same in both cases. As for the specific polysaccharides, both are salts of polyvalent, complex sugar acids, so that the charges on the particles of both must be of much the same order. If the immune reaction depended, then, on particle charges there should again be no evidence of change when some of the equally charged particles are taken from each flask and added to the other. But type I polysaccharide has thus been added to type I serum, and type III polysaccharide to type III serum, specific precipitation occurs, and the conclusion seems inescapable that the specific interaction is a chemical union. To draw a simple analogy from inorganic chemistry, there will, of course, be no precipitate when sodium *acetate* is added to barium chloride, but if sodium *sulfate* is added, specific precipitation of barium sulfate occurs at once.

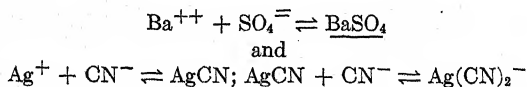
The Precipitin Reaction.—Of all the immune reactions the precipitin reaction is the simplest, but owing to analytical chemical difficulties it has only recently been possible to subject the reaction to strict quantitative study. The analytical problem can be considerably simplified by the use of the type III pneumococcus specific polysaccharide, which is a nitrogen-free substance of such definite properties as to suggest that it is in a state of high purity. Type III pneumococcus antibody may be readily obtained,²⁹ and while not pure, contains 40 to 60 per cent of specifically precipitable protein. If one admits that antibody is protein, it should be possible, by adding known amounts of nitrogen-free polysaccharide to measured amounts of antibody solution, to determine by means of nitrogen analyses the amount of antibody precipitated.³⁵ Such studies are in progress, and already a few conclusions seem possible and several practical results have been attained.

If a very small amount of type III pneumococcus polysaccharide, S, is added to a relatively large amount of antibody, A, it is found that more than 250 mg. of antibody may be precipitated for each milligram of S. As increasing amounts of S are added in proportion to the amount of A this ratio decreases, but all of the S added is precipitated, leaving antibody in excess. Finally, with increasing amounts of S, a point is reached at which there occurs for the first time a very slight excess of S. At this point there is also a small amount of antibody remaining in solution. This stage we have called the "equivalence point," and at this point the ratio of S to A is approximately 1:70. If

the amount of S is now further increased the traces of A in solution are first precipitated, but with relatively large amounts of S, the precipitate formed becomes less and less, until with higher concentrations of S no precipitation takes place. The colloid chemist would say that the precipitate is "peptized" by the excess of S, but we have confirmed Arrhenius' belief in a soluble compound and shown that in this inhibition zone new, more soluble compounds are formed in which the most soluble contains one more molecule of S than the less soluble phase. The findings may be schematized by the following equations:



in which SA_4 represents the limiting compound formed in the region of excess antibody, SA the composition of the precipitate at the "equivalence point," and S_xA the composition of the most soluble material in the inhibition zone. Each equation can be shown to represent a reversible equilibrium, and since the antibody-sodium chloride complex is ionized and S is the salt of a highly ionized polyvalent acid, the equilibria appear to be ionic and the application of the mass law seems justified. The precipitin reaction between S and its homologous antibody is thus no different in principle from an inorganic precipitation such as the specific reaction we have seen between barium and sulphate ions. Nor does one have to search far for at least a partial analogy to the inhibition zone if we recall that the insoluble silver cyanide is soluble in an excess of potassium cyanide solution. The equations are:



Thus one need not be limited as by the colloid theory to names such as adsorption, hydrophilic, hydrophobic, or peptization, which are descriptive but difficult to translate into quantitative terms. Nor need one be limited to the analogy of the union of a weak acid with a weak base, which formed the point of departure of Arrhenius' formulation of immune reactions,³⁶ for the multivalence of A and S with respect to each other is emphasized and quantitatively accounted for. The failure of the older theory of antigen-antibody union to do this was pointed out many years ago.³⁷

The Coexistence of Antigen and Antibody in Body Fluids.—Several immunological puzzles of long standing may be accounted for by treatment of the precipitin reaction in this fashion according to the laws of classical chemistry. Take, for example, the difficulty of explaining the coexistence of antigen and antibody in the body fluids. At the equivalence point, at which the equilibrium may be represented

by $\frac{[S][A]}{[SA]} = K$, free A and free S are in equilibrium with the saturated solution of the insoluble compound SA, and either is precipitated on the addition of a small amount of the other, just as addition of either ion to a saturated solution of a sparingly soluble salt produces a fresh precipitate. According to this view, the amount of A and S in solution should depend upon the equilibrium constant and would vary with every system studied. Thus in the egg albumin-antibody reaction it was reported that the amounts of antigen and antibody at the equivalence point were too small to detect.³³ On the other hand, the workers studying the hemoglobin-antibody reaction³⁴ found such large amounts of both components in equilibrium with the precipitate that they were unable to interpret their results. However, a dissociation constant may be deduced from their data, and this accounts satisfactorily for their findings.

The Danysz Phenomenon.—Another immunological puzzle which can now be explained is the Danysz phenomenon, and one may even predict for any given antibody solution how much antigen will be left over if the amount necessary to reach the equivalence point is added in definite fractions instead of all at once. A glance at Fig. 20 will make this clear. The curve shows the amount of antibody precipitated from an antibody solution by varying amounts of S.

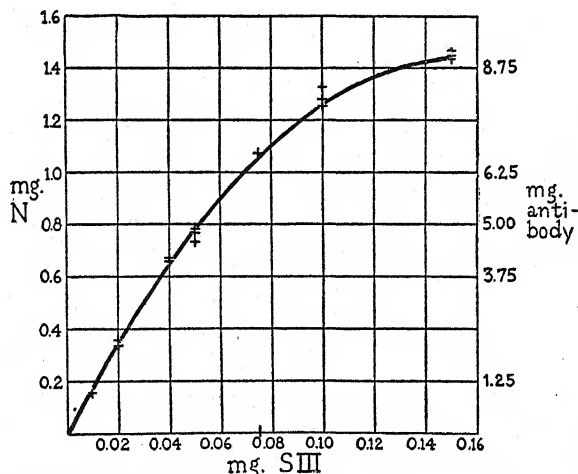


Fig. 20.—(Reprinted from *Medicine*, 12, 279 (1933); *Harvey Lectures*, 28, 184 (1933)).

At the right is the equivalence point, at which S just begins to show in slight excess. S, 0.15 mg., and 9 mg. of antibody are thus equivalent if the S is added at one time. Let us, however, first add one half of the amount of S, or 0.075 mg. Under these conditions antibody is in relative excess, and as we have seen in our previous study, the precipitate will not have the composition SA, but will be some-

where in the range SA_4-SA . From the graph, the actual amount of antibody precipitated is seen to be 6.7 mg. But this is three quarters of the antibody precipitated by the entire amount of S when added at once, and there is still one half of the S left over. This will, of course, be far more than necessary to reach the equivalence point, and the excess can be calculated.* We have, then, given a simple interpretation of the Danysz phenomenon in terms of the laws of classical chemistry, whereas it is often cited in illustration of the colloidal or adsorptive nature of immune reactions. Indeed, the reaction cannot in this case be one of adsorption, for if the fractional amount of S distributed itself over the surface of the antibody, removal of the precipitate would remove all the antibody. But we have seen that a definite portion of antibody is left over, and in order to precipitate this from the supernatant a new and definite amount of S must be added.

Microdetermination of Antigen.—The method illustrated in Fig. 20 is also of interest in another connection, since it provides a means for the quantitative microdetermination of any specific polysaccharide or antigen which can be obtained in a state of purity. All that is necessary is to calibrate an antibody solution or serum with known amounts of polysaccharide or antigen, taking care always to have an excess of antibody, so that all of the polysaccharide or antigen will be precipitated. Nitrogen is then determined in the washed precipitate by the micro-Kjeldahl method and a curve is constructed by plotting the total nitrogen precipitated against the quantity of polysaccharide or antigen used. Amounts of specific polysaccharide as low as 0.01 mg. may be determined in this way with a fair degree of accuracy, since the amount of antibody precipitated is many times that of the hapten or antigen in combination.³⁹ Figure 20 (p. 397) was arrived at in this way. The crosses represent the checks actually obtained.

Determination of Antibody.—But it is not only the antigen or hapten for which a quantitative analysis may now be made. The antibody titer of a serum has usually been given in terms of the highest dilution at which it will agglutinate, hemolyze, or precipitate the antigen, in terms of the volume of toxin it will neutralize, in terms of mouse protection—all relative and often inaccurate measures giving no idea whatsoever of the actual mass of antibody involved. If, however, it is admitted that antibodies are proteins, an absolute determination of antibody is now possible. Let us recall that at the equivalence point the specific precipitate SA is in equilibrium with small amounts of A and S (see p. 397). Clearly in order to obtain the maximum amount of specifically precipitable antibody it is necessary to add a somewhat greater amount of S, so that the excess will combine with and precipitate as much as possible of the small amount of A remaining in solution. The conditions are thus established for the precipita-

* Reprinted from *Medicine*, 12, 279 (1933); *Harvey Lectures*, 28, 184 (1933).

tion from any serum of the maximum amount of specifically precipitable antibody, and this has been made the basis of a quantitative method for the determination of precipitin.^{40, 41} The method consists in the addition of a *slight* excess of specific polysaccharide or antigen to an immune serum or antibody solution and an analysis of the washed precipitate by the micro-Kjeldahl method for nitrogen. Deduction of the amount of nitrogen due to the hapten or antigen and multiplication by the factor for protein gives milligrams of specifically precipitable antibody for the volume of serum taken. This may be as little as 0.5 cc. in the case of potent sera. The micro-Kjeldahl method had previously been used for the analysis of specific precipitates,⁴² but the conditions for the maximum precipitation of antibody were not at that time understood.

The first application of this method was in an attempt to determine whether or not mouse protection and specific precipitation ran parallel in type I antipneumococcus horse sera. Eleven sera were studied and it was found that they contained amounts of specifically precipitable protein ranging from 0.7 to 9.7 mg. per cubic centimeter.* Parallel mouse protection tests ranged from 50 to 1600 units. When the sera were arranged in the order of increasing mouse protection it was found that they were also in the order of increasing amounts of specifically precipitable antibody per cubic centimeter. Seven milligrams per cubic centimeter corresponded roughly to 1000 mouse protective units. With a very much larger number of sera, the high degree of correlation between mouse protection and the maximum amount of specifically precipitable protein was confirmed in another laboratory,⁴³ so that at least in the case of type I antipneumococcus sera it would seem reasonable to substitute the analytical method for the time-consuming, expensive and difficult mouse protection test.

The next application of the method was in the quantitative study of an antigen-antibody system. In this case, since antigen and antibody are both considered proteins, it became necessary to distinguish between antigen nitrogen and antibody nitrogen in the specific precipitate. This had already been accomplished in the determination of hemoglobin and total nitrogen in the hemoglobin-antibody precipitate, and also in the analysis of iodoalbumin-antibody precipitates for iodine and nitrogen.⁴² The simplest method, however, seemed to be the colorimetric determination of the amount of an azoprotein precipitated by its homologous antibody. For this purpose the yellow azoproteins such as those previously discussed seemed too light in shade, and accordingly the R-salt-azobenzidine-azocrystalline egg albumin derivative was made (see Diagram *d* on p. 390). This has an intense red color and may be determined colorimetrically in dilutions even greater than 1 to 100,000. The specific precipitate with its homologous antibody

* The deacetylated type I polysaccharide was used in these tests. Had the acetyl polysaccharide been used, the amounts of nitrogen precipitated would probably have been about one half again as great.

varies from pink to deep red, depending on the relative proportions of the reactants, and each component may be determined separately in the precipitate or in the supernatant, the antigen by comparison with a standard solution, and the antibody as previously described. In this way it has been possible to show that antigen and antibody in this system, too, are multivalent with respect to each other—that is, that the composition of the precipitate varies according to the relative proportions of the reactants. Thus the precipitin reaction between a complete antigen and its homologous antibody proceeds in the same manner as that between a specific polysaccharide and its antibody, the differences being solely numerical.³³

The details of the quantitative method for the determination of precipitins follow; the description is that for the dye-antidye system:⁴¹

In the original method⁴⁰ the precipitate formed by the specific polysaccharide of type I pneumococcus and its homologous antibody was washed only once with a 1: 20,000 solution of specific polysaccharide in saline. It has since been found³⁹ unnecessary to add the specific carbohydrate to the washing fluid, nor does dissociation occur in the azoprotein-antibody system when the precipitate is washed in the cold with 0.9 per cent saline alone. Two washings are also necessary³⁹ if complete removal of nonspecific serum proteins is desired.

Depending on the intensity of a preliminary rapid qualitative test with 1: 10,000 dye, amounts of serum ranging from 0.5 to 4 cc. are used. A number of sera may be analyzed at one time. The sera should be measured in duplicate with accurately calibrated pipets into wide agglutination tubes (10 mm. inside diameter x 75 mm.) or Wassermann tubes, depending on the amount of serum used. Blanks should also be run in duplicate and saline added to these instead of dye solution. If less than 2 cc. of serum have been used the volume should be made up to 2 cc. with saline. An amount of a 1: 1000 solution of the dye in saline is then added sufficient to provide a *slight* excess of the dye. Not more than 0.10 cc. should be added in the case of sera containing less than 0.1 mg. of precipitable antibody per cubic centimeter (3 to 4 cc. samples), and the volume of stronger sera should be chosen so that not more than 0.2 or 0.3 cc. of the 1: 1000 dye solution need be used. Calibrated pipets are not necessary for the dye. The tubes are plugged and the contents carefully and thoroughly mixed by a rotary motion imparted by drawing the fingertips rapidly and repeatedly diagonally down the side of the tube. The tubes are set in the water bath and may be centrifuged, if desired, as soon as the precipitate begins to settle, in order to make sure that an excess of dye has been added. If the supernatant is not definitely pink as compared with a blank on the same serum, 0.05 to 0.1 cc. more of the dye dilution should be added at a time until a definite excess is present. Supernatants should not contain so large an excess as to be definitely red, as many sera show a marked inhibition zone beginning with surprisingly low con-

centrations of antigen in excess. If the supernatants are red, more serum should be added to the determinations and blanks, or a new analysis started. In the case of weak sera the precipitates are often very slow in forming. The tubes are allowed to stand two hours in the water bath at 37° C. and over night in the ice-box, or else may be left at room temperature for a period and then over night in the ice-box, or may be immediately put into the ice-box for eighteen to forty-eight hours. The rabbit antisera tested in this laboratory have given identical results under these conditions, except that the precipitate forms at a slower rate in the cold. The systems tested have been dye-antidye, egg albumin and its homologous antibody, and type III pneumococcus specific polysaccharide and rabbit type III pneumococcus antiserum, so that in these cases there would seem to be no basis, except on the ground of increased speed of reaction, for the current immunological practice of allowing precipitin tests to stand two hours at 37° C. before placing in the ice-box over night. This observation does not apply to immune horse sera, in which the differences found are being subjected to closer study.

After the tubes have stood over night they are centrifuged in the refrigerating centrifuge or in carriers containing ice-water for fifteen to twenty minutes at about 1500 revolutions per minute. The supernatants are then carefully decanted and the tubes are inverted, allowed to drain five minutes, and the mouths wiped with filter paper. The tubes are placed in ice-water and 0.5 cc. of ice-cold saline is added to each. The contents are mixed as before and the red precipitates should be thoroughly disintegrated in order to insure as complete removal of nonspecific protein as possible. The tubes are then rinsed down with 1.5 cc. of ice-cold saline in the case of the small tubes and 2.5 cc. for the Wassermann tubes and again mixed.

Blank tubes which show no whirl when the contents are mixed at the first or second washing may be discarded, as under these conditions the blank to be deducted in the nitrogen determination is no larger than that on the reagents alone.

While the tubes are standing in ice-water for one-half hour the original supernatants should be tested as a control for the presence of a slight excess of dye. To one 0.5 cc. portion of the mixed duplicate supernatants from each serum is added 0.1 cc. of a 1: 10,000 dye solution, to another, 0.2 to 0.3 cc. of the blank supernatant from a serum which has given a heavy precipitate. There should, of course, be no precipitate in the tube to which additional dye was added, while the tube to which antiserum was added should show a slight turbidity within two hours or at least a slight precipitate on standing over night in the ice-box.

After one-half hour in the cold the washed precipitates are centrifuged, decanted, and drained as before, and again washed as above with 1.5 or 2 cc. of chilled saline, depending on the size of tube. After standing for one-half hour in ice-water, the tubes are finally cen-

trifuged, decanted, and drained. The precipitates are covered with 1.5 to 2 cc. of water, loosened from the bottom of the tube by rotating as before, and dissolved by the addition of 2 to 3 drops of N sodium hydroxide solution.

The amount of azoprotein in the precipitate may be determined by making up the volume of the solution to 5 or 10 cc. and comparing the color with that of a known solution of the dye containing the same amount of alkali. Since the ratio of dye to antibody in the precipitate has been found in this laboratory to average 1: 8 when a slight excess of antigen is present, the determination of antigen in the precipitate may be omitted if it is desired to accept this figure. In the application of the method to any other antigen it would be necessary to determine this ratio for the system used.

The solution of the precipitate is rinsed quantitatively into a micro-Kjeldahl flask and the nitrogen determined by any standard procedure. The Pregl method, slightly modified, was used in the present work. Nitrogen found $\times 6.25$ = specifically precipitated protein in the sample.

Total protein minus antigen protein $\left(\frac{\text{total}}{9}\right)$ = precipitable antibody.

With the aid of the quantitative method it has been possible to follow antibody production in its various stages in animals.⁴¹ One thing which it has brought out clearly is the astounding disproportion between the amount of antigen injected and the amount of antibody produced in the animal. While this has long been realized in a qualitative way, it is now found that the serum of reactive rabbits may contain as much as 80 to 110 mg. of precipitin per milligram of antigen injected. Since there is antibody in the tissues as well, this may be taken as supplementing the other evidence quoted against the actual entrance of specific antigen fragments into the antibody molecule.

The quantitative method has also been used in a study of the egg albumin-antibody system³⁸ and a simple modification introduced which is applicable to that system. With its aid it was shown that the circulating antibody alone accounts quantitatively for the rapid disappearance of egg albumin injected into the circulation of immunized rabbits, and the method has also been used for the determination of blood volume in rabbits.⁴⁵

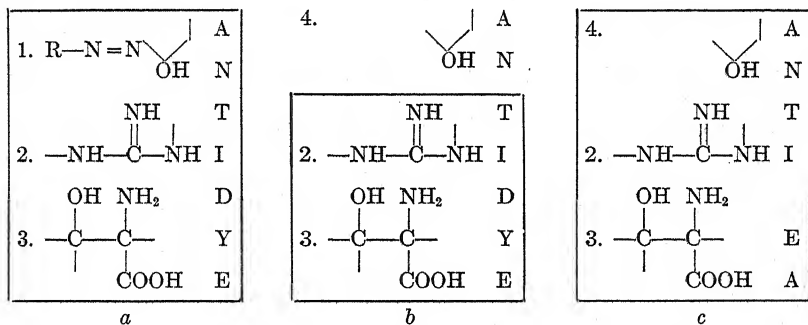
The method described above in detail may also be directly applied to the egg albumin-antibody system, since the total amount of egg albumin added is precipitated as far as the equivalence point. Beyond the equivalence point the excess of antigen may be determined in the supernatant by the method given on page 398 (see also Fig. 20), so that at all proportions of egg albumin and antibody the amount of antigen in the precipitate is either known or may be calculated. The writer has found the composition of the precipitate to vary according to the proportions of antigen and antibody, just as in the other cases studied, and the ratio at the equivalence point to average about 1: 11.*

* Unpublished experiments.

Application of the method along the same lines to other antigen-antibody systems would seem reasonable.

Explanation of Cross-precipitation.—The use of the quantitative precipitin method has also led to a clear, simple explanation of at least a few instances of cross-precipitation. It was found, for example, that the dye antigen described above, when rigorously purified, seldom yielded precipitates in anti egg albumin sera. However, suitable amounts of crystalline egg albumin precipitated antisera obtained on injection of the purified dye protein, and with very large amounts of egg albumin, far greater than sufficient to throw the homologous reaction into the inhibition zone, nearly all of the antibody in some anti-dye sera could be precipitated. That the antibody reacting with egg albumin was actually antidye was shown (1) by the difference in form and direction of the curve obtained as in Fig. 20 from that characteristic for homologous reactions, and (2) the absence of precipitation in certain sera when very small quantities of egg albumin were added, although precipitation occurred with larger amounts.⁴⁶ At all points free egg albumin could be detected by the addition of anti egg albumin, so that the cross-precipitation would be most simply explained on the basis of a loose, highly dissociated combination between the egg albumin and antidye, as opposed to the firm, almost undissociated union between dye protein and antidye, or egg albumin and anti egg albumin.

This may, perhaps, be more easily visualized in the following diagrams:



Grouping 1, in accordance with Landsteiner's findings, is the dominant antigenic group in the dye protein, while 2 and 3 are other arbitrarily chosen groups of which the steric configuration is characteristic for egg albumin. The dominant reactive group on the antidye molecule would be one evoked in the animal by virtue of the dominant antigenic group.³⁴ The resulting dye-antidye combination might therefore be represented by Diagram *a*, and would be expected to be relatively firm and undissociated. Other less important groups on the antibody molecule might be expected to result from the groupings 2 and 3, characteristic of the egg albumin portion of the dye molecule. Thus crystalline egg albumin, with its dominant antigenic group 4,

arbitrarily chosen in analogy with 1, might be expected to react with antidye by virtue of groupings 2 and 3. The union could reasonably be expected to be a relatively weak, highly dissociated one, since there would be no anti-4 in the antidye, and the egg albumin contains no grouping 1 to react by virtue of the anti-1 present. This is represented in Diagram *b*. Again, crystalline egg albumin, with its dominant antigenic group 4, would be expected to form the observed firm, undissociated union with anti-egg albumin, containing anti-4, as represented in Diagram *c*.

The views on which this interpretation is based are given in references 46, 47 and 48. It is already evident that they may be applied in other cases of cross-precipitation. It is equally evident, however, that in many cases it is necessary to assume that several antibodies are formed, and that only a portion of these cross-react.

Bacterial Agglutination.—A treatment similar to that given the precipitin reaction may be applied to another immune reaction; namely, bacterial agglutination. There is a close parallel between the successive stages of the precipitin reaction and the agglutination of type specific pneumococci,⁴⁹ so that bacterial agglutination, as foreshadowed by earlier work,⁵⁰ is merely a precipitin reaction at the surface of bacteria, and, subject to this restriction, is governed by the same laws. Thus by the use of a washed bacterial suspension, containing a known amount of nitrogen, agglutinins may be determined in certain sera by the increase in the amount of nitrogen after agglutination,* provided the suspension is in excess and the increase in nitrogen is large enough to be within the range of the micro-Kjeldahl method.⁵¹ The method also brings to light, with an exactness which had not previously been attained,⁵² the quantitative correspondence of agglutinin and precipitin in the special case of a pneumococcus antibody solution.⁵¹

The Toxin-antitoxin Reaction.—The toxin-antitoxin reaction, involving as it does the direct union of antigen and antibody, is very similar to the precipitin reaction and is, indeed, often accompanied by flocculation. That the precipitate is derived mainly from the serum pseudoglobulin has been previously mentioned.† The chief difficulties in the way of a chemical study of the reaction lie in the lack of chemical knowledge of toxins, for pure toxin has never been isolated, and in the cumbersome mechanism of the animal tests for toxin and antitoxin. The use of the flocculation reaction for the measurement in relative terms of either component now seems feasible, the more so as it has been found that many of the inconsistencies between the flocculation and animal tests vanish if the mixtures are allowed to stand long enough to come to equilibrium before injection into animals.⁵³

* Or by the decrease in the nitrogen of the supernatant.

† Also *Brit. J. Exp. Path.*, 12, 182 (1931), in which exception is taken to a mass law interpretation of the precipitin reaction. A similar objection is also made in reference 34A.

The analogy between the precipitin and toxin-antitoxin reactions is also clearly shown in the Danysz phenomenon, according to which a volume of toxin, added all at once to an equivalent amount of antitoxin is just neutralized by the antitoxin, but proves excessive if added fractionally. To the discoverer⁵⁴ this was clear evidence that toxin and antitoxin could combine in more than one proportion, just as was found for antigen and antibody in the precipitin reaction.³⁵ Nevertheless, the Danysz effect has come to be quoted either as evidence for the adsorptive nature of toxin-antitoxin union, or as so puzzling that one worker in the field⁵³ finds himself unable to offer any explanation. The known facts are, however, exactly what would be predicted if toxin and antitoxin could combine, as Danysz maintained, in multiple proportions, and if the equilibrium at the equivalence point were reversible, as has been shown for the analogous precipitin reaction and, in one instance, for a toxin-antitoxin reaction.³⁶ And in much the same way many of the difficulties recently encountered by students of the toxin-antitoxin reaction might have been predicted and may be simply explained if the analogy between the precipitin and toxin-antitoxin reactions be assumed to hold.*

* * * * *

In this chapter the writer has tried to show how chemistry has made steps toward giving more definite meaning to the concepts antigen and antibody and providing a better understanding of the mechanism of the immune reactions in which they participate. The introduction of known chemical groups into the protein molecule, with its consequent sensitive control of specificity, and the recognition of the large part played by polysaccharides in bacterial specificity have served to emphasize the essentially chemical and ultimately minutely determinable basis of biological specificity, and have simplified and clarified relationships and provided powerful aids for further progress. With highly purified antibody close at hand, and with plausible theories as to its formation, the many problems connected with antibodies should be well on their way toward solution. With these newer aids it has been possible to obtain strong evidence of the chemical union of antigen or hapten with antibody in multiple proportions, and to express this union in terms of the laws of classical chemistry. On this foundation there are now accessible new and absolute quantitative methods which should be useful tools in the acquisition of a final complete understanding of immune processes.

MICHAEL HEIDELBERGER.

* Cf., for example, Heidelberg, M., and Kendall, F. E., *Science*, **71**, 511 (1930) —a point of view not accepted by Ramon, G., *Compt. rend. soc. biol.*, **104**, 938 (1930); **105**, 173; or Schmidt, S., *ibid.*, **105**, 94. The latter has, however, modified his original views: (53), also Madsen, T., and Schmidt, S., *Z. Immunf.*, **65**, 357 (1930).

REFERENCES

1. Wells, H. G.: *Chemical Aspects of Immunity* (1929).
2. Hopkins, S. J., and Wormald, A.: *Biochem. J.*, **27**, 740, 1706 (1933).
3. Erlenmeyer, H., and Berger, E.: *Biochem. Z.*, **252**, 22 (1932); **255**, 429, 434 (1933); **256**, 196 (1933); *Helv. Chim. Acta*, **16**, 733 (1933).
4. Dochez, A. R., and Avery, O. T.: *J. Exp. Med.*, **26**, 477 (1917).
5. Zinsser, H., and Parker, J. T.: *J. Exp. Med.*, **37**, 275 (1923).
6. Heidelberger, M., and Avery, O. T.: *J. Exp. Med.*, **38**, 81 (1923).
7. Avery, O. T., and Heidelberger, M.: *J. Exp. Med.*, **42**, 367 (1925).
8. Heidelberger, M., Goebel, W. F., and Avery, O. T.: *J. Exp. Med.*, **42**, 727 (1925).
9. Heidelberger, M., and Goebel, W. F.: *J. Biol. Chem.*, **70**, 613 (1926); **74**, 613 (1927).
10. (A) Avery, O. T., and Goebel, W. F.: *J. Exp. Med.*, **58**, 731 (1933); (B) Pappenheimer, A. M., Jr., and Enders, J. T.: *Proc. Soc. Exp. Biol. Med.*, **31**, 37 (1933).
11. Avery, O. T.: *Naturwissenschaften*, **21**, 777 (1933).
12. Heidelberger, M., and Kendall, F. E.: *J. Biol. Chem.*, **96**, 541 (1932).
13. Haworth, W. N., and Machemer, H.: *J. Chem. Soc.*, 2270 (1932); Hirst, E. L., Plant, M. M. T., and Wilkinson, M. D.: *J. Chem. Soc.*, 2375 (1932).
14. Heidelberger, M., and Kendall, F. E.: *J. Exp. Med.*, **57**, 373 (1933).
15. Landsteiner, K., and van der Scheer, J.: *J. Exp. Med.*, **56**, 399 (1932).
16. Goebel, W. F., and Avery, O. T.: *J. Exp. Med.*, **50**, 521 (1929); Avery, O. T., and Goebel, W. F.: *J. Exp. Med.*, **50**, 533 (1929); see also later papers.
17. See also Landsteiner, K., and van der Scheer, J.: *J. Exp. Med.*, **48**, 315 (1928); **50**, 407 (1929).
18. Goebel, W. F., and Avery, O. T.: *J. Exp. Med.*, **54**, 431 (1931); Avery, O. T., and Goebel, W. F.: *J. Exp. Med.*, **54**, 437 (1931).
19. Dubos, R., and Avery, O. T.: *J. Exp. Med.*, **54**, 51 (1931); Avery, O. T., and Dubos, R.: *J. Exp. Med.*, **54**, 73 (1931).
20. Francis, T., Jr., and Tillett, W. S.: *J. Exp. Med.*, **52**, 573 (1930).
21. Zozaya, J.: *J. Exp. Med.*, **55**, 325 (1932); **57**, 21 (1933); Freund, J.: *Science*, **75**, 418 (1932). See, however, Jacobs, J.: *J. Exp. Med.*, **59**, 479 (1934).
22. Landsteiner, K., and Levene, P. A.: *J. Immunol.*, **14**, 81 (1927).
23. Anderson, R. J.: *Physiol. Rev.*, **12**, 141 (1932).
24. The case for lipid haptens is very ably summed up by Rudy, H.: *Kolloid chem. Z.*, **65**, 356 (1933).
25. Cf. Bossa, G.: *Z. Hyg. Infektskr.*, **114**, 77 (1932); Kligler, I. J., and Olitzky, L.: *Brit. J. Exp. Path.*, **13**, 237 (1932); Andrewes, C. H., and Elfjord, W. J.: *Brit. J. Exp. Path.*, **14**, 367, 376 (1933).
26. Krueger, A. P.: *J. Gen. Physiol.*, **13**, 553, 557 (1930); **14**, 493 (1931); Krueger, A. P., and Northrop, J. H.: *J. Gen. Physiol.*, **14**, 223 (1931); Schlesinger, M.: *Z. Hyg. Infektskr.*, **114**, 136 (1932).
27. Schlesinger, M.: *Biochem. Z.*, **264**, 6 (1933).
28. Dean, H. R.: *A System of Bacteriology in Relation to Medicine* (1931), volume 6.
29. Felton, L. D.: *J. Infec. Dis.*, **43**, 543 (1928) and earlier papers.
30. Felton, L. D.: *J. Immunol.*, **22**, 453 (1932).
31. Marrack, J. R., and Smith, F. C.: *Proc. Roy. Soc. (London)*, **106B**, 1 (1930).
32. Doerr, R., and Friedli: *Arch. Dermatol. Syphilis*, **151**, 11 (1926); Berger, E., and Erlenmeyer, H.: *Z. Hyg. Infektskr.*, **113**, 79 (1931); Hooker, S. B., and Boyd, W. C.: *J. Immunol.*, **23**, 465 (1932).
33. Heidelberger, M., and Kendall, F. E.: *Science*, **72**, 252, 253 (1930).
34. (A) Breinl, F., and Haurowitz, F.: *Z. physiol. Chem.*, **192**, 45 (1930); (B) Mudd, S.: *J. Immunol.*, **23**, 423 (1932).
35. Heidelberger, M., and Kendall, F. E.: *J. Exp. Med.*, **50**, 809 (1929).
36. Arrhenius, S.: *Immunochemistry* (1907).
37. Fleischmann, P., and Michaelis, L.: *Biochem. Z.*, **3**, 425 (1907).
38. Culbertson, J. T.: *J. Immunol.*, **23**, 439 (1932).
39. Heidelberger, M., and Kendall, F. E.: *J. Exp. Med.*, **55**, 555 (1932).
40. Heidelberger, M., Sia, R. H. P., and Kendall, F. E.: *J. Exp. Med.*, **52**, 477 (1930).
41. Heidelberger, M., and Kendall, F. E.: *J. Exp. Med.*, **58**, 137 (1933).
42. Wu, H., Cheng, L. H., and Li, C. P.: *Proc. Soc. Exp. Biol. Med.*, **25**, 853 (1927); **26**, 737 (1929).

43. Felton, L. D.: *J. Immunol.*, **21**, 341 (1931).
44. Culbertson, J. T., and Seegal, B. C.: *Proc. Soc. Exp. Biol. Med.*, **29**, 209 (1932).
45. Culbertson, J. T.: *Proc. Soc. Exp. Biol. Med.*, **30**, 102 (1932).
46. Heidelberger, M., and Kendall, F. E.: *J. Exp. Med.*, **59**, 519 (1934).
47. Landsteiner, K., and van der Scheer, J.: *J. Exp. Med.*, **42**, 123 (1925); Hooker, S. B., and Boyd, W. C., *J. Immunol.*, **25**, 61 (1933).
48. Bergmann, M.: *Science*, **79**, 439 (1934).
49. Francis, T., Jr.: *J. Exp. Med.*, **55**, 55 (1932).
50. Joos, A.: *Z. Hyg. Infektskr.*, **40**, 203 (1902); Northrop, J. H., and de Kruif, P.: *J. Gen. Physiol.*, **4**, 639, 655 (1922); Shibley, G. S.: *J. Exp. Med.*, **40**, 453 (1924).
51. Heidelberger, M., and Kabat, E. A.: *Proc. Soc. Exp. Biol. Med.*, **31**, 595, 597 (1934); *J. Exp. Med.*, **60**, 643 (1934).
52. Zinsser, H.: *J. Immunol.*, **18**, 483 (1930).
53. Schmidt, S.: *Z. Immunf.*, **67**, 197 (1930).
54. Danysz, J.: *Ann. inst. Pasteur*, **16**, 331 (1902).

CHAPTER XV

BLOOD

BLOOD AND LYMPH

As Claude Bernard (1878) pointed out in his last and most monumental work, the evolution of the highest forms of life has been possible owing to "the liquid *milieu intérieur* formed by the circulating organic liquid which surrounds and bathes all the tissue elements; this is the lymph or plasma, the liquid part of the blood which, in the higher animals, is diffused through the tissues and forms the ensemble of the intercellular liquids and is the basis of all local nutrition and the common factor of all elementary exchanges." L. J. Henderson (1928) writes "this theory of the constancy of the *milieu intérieur* was an induction from relatively few facts, but the discoveries of the last fifty years and the introduction of physicochemical methods into physiology have proved that it is well founded."

Functions of Blood.—1. It is the seat of important physiologic processes.

2. It provides fluid and maintains the osmotic relations of the cells and tissues.

3. It conveys foodstuffs (sources of energy) and oxygen for the biological combustions whereby their energy is made available.

4. It also provides materials for building and repairing the tissues.

5. It removes metabolites including carbon dioxide, lactic acid, and other "waste products."

6. It maintains the all-important acid-base equilibrium.

7. It aids in dissipating the heat evolved during cellular activity and maintains the equable body temperature of warm-blood animals.

8. By transporting hormones and in other ways it functions as the great chemical "integrative mechanism" whereby the activities of the different parts of the body are coordinated for the effective unity of action and well-being of the whole.

9. By clotting and associated phenomena it guards against hemorrhage.

10. It contributes cellular and humoral factors to the immunological defense mechanisms.

Functions of Lymph.—If we draw an analogy between the circulatory systems of the body and that of a modern city, we may liken the arterial system to the water supply and the venous drainage to the sewage system. The lymphatic system would then be likened to the storm water drainage system—a mechanism for taking care of sudden emergencies. The earlier work of Ludwig and his pupils has been recently confirmed by Drinker and Field who find very little lymph drains from the subcutaneous tissues at rest, and only a small amount

after exercise and passive massage. The flow is increased, however, under conditions of edema formation. The modern view (Sabin *et al.*) is that the lymphatic system, with a few regional exceptions (MacCallum) resembles the blood vascular system in being a "closed system."

Functions of Tissue and Serous Fluids.—The tissue fluids interpose a variable amount of "buffering" menstruum between the cells and the blood and lymphatic systems. In the areolar tissues rather considerable quantities are present in the groundwork of interstitial substance, which probably exists in the form of a dilute colloidal "gel" (Clark and Clark, 1932). In the lungs, liver, kidneys, etc., the intervening fluid is scanty. In the pleural, pericardial, peritoneal (and scrotal) "cavities" there is normally little free fluid owing to the pressure relationships, but the quantity may be greatly increased in abnormal states. Considerable quantities of fluid are present in the cerebrospinal cavities, the synovial cavities of joints and bursae (sometimes), and the chambers of the eye, but in these sites the fluid subserves special functions.

Composition of the Blood.*—Blood comprises a protein-rich fluid portion or *plasma* in which are suspended the various "formed elements" including the red blood corpuscles (*erythrocytes*), white blood cells (*leukocytes*), and the *platelets* (or *thrombocytes* of the lower animals). If clotting is prevented, usually by "decalcifying anticoagulants" (see *blood coagulation*), blood may be centrifuged in an hematocrit and the corpuscle and plasma volumes compared. The corpuscles normally constitute about 45 ± 5 per cent of the total blood volume, but the ratio is probably not the same in all parts of the circulation.

Specific Gravity, etc.—The specific gravity of whole blood is normally 1.054–1.06, and of plasma, $1.026 \pm .002$ (Peters and van Slyke). The erythrocyte specific gravity is approximately 1.09. Water constitutes 90–92 per cent of the plasma weight, and 64–65 per cent of the corpuscles. Plasma contains 8–9 per cent of solids, of which (a) the proteins make up 7–7.1 per cent (Salvesen; Moore and van Slyke), (b) lipoids form 0.7 per cent, (c) inorganic constituents, 0.75 per cent, and (d) miscellaneous organic constituents, 0.15 per cent.

The *osmotic pressure* of plasma is about 6.5 atmospheres and sodium chloride accounts for most of it. The *colloid osmotic pressure*, which is so important for the physiologic transfer of fluids (*e. g.*, tissues \rightleftharpoons blood \rightleftharpoons urine) is mainly that of the plasma proteins (*q. v.*). Krogh (1929) estimated the value to be 380 mm. of water (27.94 mm. of mercury) which Fellows (1931) confirmed. According to molecular-weight computations by Svedberg, and by Adair and Robinson (see (*plasma proteins*)) albumin should be about 2.5 times as active, osmotically, as globulin. Govaerts (1925–'26), however, found the proportion to be nearly 4:1 in experimental determinations.

* Hemoglobin chemistry is discussed in Chapter XVIII.

TABLE 1
COMPOSITION OF HUMAN BLOOD

| Constituent. | Normal range mg. per 100 cc. ¹ | Pathologic conditions in which increases (unless otherwise noted) may be encountered. |
|---|---|---|
| Total solids, per cent. | 19-23 | Anhydremia. Low in hydremic plethora and anemia. |
| Total proteins (serum) per cent. | 6.5-8.2 | See above. Low in nephritis with edema (nephrosis). |
| Albumin (serum) per cent. | 4.6-6.7 | Muscular activity. Low in nephrosis. |
| Globulin (serum) per cent. | 1.2-2.3 | Syphilis, pneumonia, uremia, anaphylaxis. |
| Fibrinogen (plasma) per cent. | 0.3-0.6 | Pneumonia, septicemia. Low in liver diseases and cachexias. |
| Hemoglobin per cent (Haden)..... | 15.6 | Polycythemia. Low in primary and secondary anemia, chlorosis. |
| Total nitrogen, per cent. | 3.0-3.7 | Varies chiefly with proteins (albumin globulin, hemoglobin). |
| Nonprotein nitrogen..... | 25-35 | Nephritis, eclampsia, etc. See Urea nitrogen. |
| Urea nitrogen..... | 10-15 | Chronic and acute nephritis, metallic poisoning, cardiac failure, intestinal or prostatic obstruction, some infectious diseases. Relatively low in nephrosis. |
| Uric acid..... | 2-3.5 | Nephritis, gout, arthritis, eclampsia. |
| Creatinine..... | 1-2 | Nephritis. |
| Creatine..... | 3-7 | Terminal nephritis. |
| Amino-acid nitrogen..... | 5-8 | Leukemia, acute yellow atrophy of the liver, severe nephritis. |
| Ammonia nitrogen..... | 0.1-0.2 | Terminal interstitial nephritis. |
| Undetermined nitrogen..... | 4-18 | Eclampsia. |
| Glucose..... | 70-100 | Diabetes, pregnancy, severe nephritis. |
| Total fatty acids..... | 290-420 | Diabetes, nephritis. |
| Cholesterol..... | 150-190 | Diabetes, nephritis, nephrosis, biliary obstruction, pregnancy. Low in pernicious anemia. |
| Lipoid phosphorus (lecithin)..... | 12-14 | Diabetes, nephritis, pregnancy. In anemia, low in plasma, high in cells. |
| Total acetone bodies (as acetone)..... | 1.3-2.6 | Diabetes. |
| Acetone + aceto-acetic acid (as acetone).... | 0.3-2.0 | Diabetes. |
| β -Hydroxybutyric acid (as acetone)..... | 0.5-3.0 | Diabetes. |
| Bilirubin..... | 0.1-0.25 | Biliary obstruction, hemolytic anemias. Low in secondary anemia. |
| CO ₂ capacity (plasma) volume per cent..... | 55-75 ² | Respiratory diseases, tetany. Low in diabetes, nephritis. |
| CO ₂ content (arterial blood) volume per cent. | 45-55 ² | Respiratory diseases, tetany. Low in diabetes, nephritis. |
| CO ₂ content (venous blood) volume per cent. | 50-60 ² | Respiratory diseases, tetany. Low in diabetes, nephritis. |
| O ₂ capacity volume per cent..... | 16-24 ² | Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia. |
| O ₂ content (arterial blood) volume per cent.. | 15-23 ² | Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia. |
| O ₂ content (venous blood) volume per cent.. | 10-18 ² | Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia. |
| Lactic acid..... | 5-20 | Exercise, eclampsia. |
| Phenols..... | 1-2 | Intestinal obstruction. |

TABLE 1—*Continued*

| Constituent. | Normal range mg. per 100 cc. ¹ | Pathologic conditions in which increases (unless otherwise noted) may be encountered. |
|--|---|---|
| Chlorides as NaCl..... | 450-500 | Nephritis, cardiac conditions, prostatic obstruction, eclampsia, anemia. Low in diabetes, fever, and pneumonia. |
| Sulphates, inorganic as S..... | 0.5-1.0 | Nephritis. |
| Phosphorus, inorganic as P (plasma)..... | 3-4 | Nephritis. Low in rickets. Normal values 1-2 mg., higher in children. |
| Calcium (serum)..... | 9-11 | Low in infantile tetany, severe nephritis, parathyroidectomy. |
| Magnesium (serum)..... | 2-3 | No changes noted in disease. |
| Sodium (serum)..... | 330 | Low in cases of alkali deficit. |
| Potassium (serum)..... | 16-22 | Uremia, eclampsia. |

¹ Figures express concentration in mg. per 100 cc. of whole blood unless otherwise indicated in first column.

² Figures represent weighted averages of the observations of several investigators.

The *constituents of the blood* may be listed under the following heads:

1. Water.
2. Gases—O₂, CO₂, N₂.
3. Proteins—(a) plasma: fibrinogen, globulin(s), albumin(s); (b) cell proteins, excluding hemoglobin.
4. Pigments—hemoglobin (red blood corpuscles), bilirubin (serum), etc.
5. Nonprotein nitrogenous bodies (exclusive of phosphatides)—urea, uric acid, creatine, creatinine, amino acids, ammonium salts, and a fraction known as "undetermined N."
6. Glucose, lactates, phenols, etc.
7. Cholesterol and such "lipoids" as fats, soaps, and the phosphatides (phospholipoids); together with the intermediary products of fat metabolism ("acetone bodies").
8. Inorganic salts: (a) kations—H, Na, K, Ca, Mg, Fe; (b) anions—chlorides, carbonates, phosphates, sulfates.
9. Enzymes and antienzymes—glycolase, lipase, esterase, oxidase, antiprotease, etc. (and the proteolytic and diastatic enzymes of the leukocytes).
10. Hormones and other "autocoids" (Schafer).
11. Immune bodies of various kinds.

N. B.—The factors concerned with blood coagulation probably fall into the foregoing groups.

Full accounts of the clinical significance of *blood chemistry* may be found in a number of books devoted to this field. We may mention Peters and van Slyke, Folin, de Wesselow, Myers, Beaumont and Dodds, and Hawk and Bergeim. Table 1 (after Hawk and Bergeim: *Practical Physiological Chemistry*, p. 357, 9th Ed., 1926, P. Blakiston's Son & Co., Inc., Publishers) lists the commonly accepted quantitative

data concerning the blood constituents, and the clinical conditions in which important alterations occur.

Blood Volume.—From a physiologic standpoint, the blood volume is important in maintaining the venous return which controls the heart's stroke and keeps up the systemic blood pressure upon which the circulation depends. From the biochemical point of view, the close interrelation between the water balance and the salts, proteins, and other chemical constituents of the blood must ever be borne in mind.

Methods of Determining Blood Volume.—1. Simple clinical methods, involving comparative hemoglobin estimations, red cell counts, with or without hematocrit determinations.

2. Welcker's method of direct exsanguination (refer to Fleischer-Hansen, 1930).

3. The CO-hemoglobin method introduced by Grehent and Quinquard, on animals, and applied to man by J. S. Haldane, with subsequent improvements and modifications by various workers. The principles of the method are: (a) Inhalation of a measured (safe) quantity of carbon monoxide gas, (b) determination of the amount of CO-hemoglobin by colorimetry, (c) estimation of the total amount of hemoglobin in the body, (d) calculation of total blood volume from hematocrit determinations of plasma: Cell ratio.

4. The dye method, introduced by Keith, Rowntree and Geraghty, which consists of: (a) Injecting (intravenously) a known amount of dye in solution, (b) withdrawing a blood sample in four to five minutes, (c) colorimetric determination of the dye in the plasma after centrifuging, (d) calculating plasma volume and hence total volume by a simple computation.

Without detailing the criteria of suitability of the dye chosen, we may list (as most frequently employed): Vital red, brilliant vital red, Congo red, trypan red, and blue azo dyes.

5. Meek and Gasser used acacia as the injection material.

6. Abderhalden and Schmidt employed dextrin and determined it from the optical activity of the blood.

Conditions Affecting Blood Volume.—(Erlanger, 1921; Seyderhelm and Lampe, 1925, '28; Griesbach, 1928; Rowntree and Brown, 1929). In the same individual the total blood volume remains remarkably constant over long periods, being 5 or 6 liters (10–12 pints) or (approximately) one eleventh of the body weight. Individual variations in body weight are the most important cause of blood volume differences. Surface area might be a more important theoretical consideration but the distinction does not seem to have any practical value. Age and sex differences parallel weight differences in a general way but the correspondence is not exact. Pregnancy is accompanied by a variable increase in blood volume, and a return to normal follows delivery.

Water, Salts and Foods.—Water deprivation soon causes dehydration of the tissues with lessening of the blood volume. Permeation of

the tissues and the efficient renal mechanism for water elimination (diuresis) prevents a demonstrable increase in the circulating blood fluid unless there is a very considerable intake of liquids. Salts modify the water balance by their osmotic effects and temporarily alter the blood volume. Sodium chloride and glucose (to a slight extent), by mouth, and more readily by intravenous administration, cause hydremic plethora, and subsequent diuresis owing to dilution of the plasma colloids and (to some extent), the improved renal circulation.

Blood alkalinizers, like sodium bicarbonate, favor hydremia, whereas acidifiers, like ammonium chloride, antagonize it and may even produce plasma concentration and diminished blood volume.

We may briefly summarize the rôle of water, salts and foodstuffs in the regulation of the blood volume by referring to (1) the mechanisms of intake, absorption, storage, metabolic mobilization, and elimination of water and (2) the osmotic shifts due to the "salt action" of the various dissolved substances.

Pathologic Variations in Blood Volume.—Increase in the total blood volume is termed *plethora* and it may be chiefly a plasma increase (hydremia) or a corpuscular increase (polycythemia). Decrease in blood volume is termed *oligemia* and is known as *anhydremia* if the plasma is especially involved, and *oligocythemia* (anemia) if the cell count is lessened. Only erythrocyte changes are significant in this connection. To avoid confusion with blood cell diseases Rowntree and his coworkers prefer to use the terms *normo-*, *hypo-*, and *hypervolemia* to designate normal, hydremic, or plethoric blood volumes (respectively), and to prefix the terms *simple*, *polycythemic*, and *oligocythemic* to denote normal, increased, or decreased erythrocyte counts (respectively).

Simple hypovolemia occurs in obesity and in certain types of renal edema. Polycythemic hypovolemia occurs in conditions of (1) water deprivation—*e. g.*, starvation, pyloric obstruction, etc., (2) excessive loss of water by mouth (vomiting), bowels (diarrhea), sweat (diaphoresis), or urine (diuresis), (3) excessive transudation of water into tissues (acidosis, irritant poisoning of capillaries—as in ether anesthesia, influenza, war gas poisoning, extensive burns, and various types of "shock").

Oligocythemic hypovolemia occurs in anemias and myxedema.

Hypervolemia with polycythemia occurs in the newborn, and in congenital heart disease, acclimatization to high altitudes, as well as in polycythemia rubra vera (Vaquez-Osler's disease) and to some extent in the leukemias. Hypervolemia with oligocythemia (at least relative) is encountered in splenic anemia, Banti's disease, and some cases of liver cirrhosis. Simple hypervolemia might be thought to occur in the so-called "full-blooded" individual especially when there is an accompanying cardiac or renal condition and an elevated blood pressure. Actual data are conflicting and often negative except per-

haps in the case with nephrosis or nephritis of the subacute type (with edema).

Blood Viscosity.—Blood viscosity is of considerable physiologic importance in determining the amount of frictional resistance to the passage of blood through the smaller arterioles, although the vessel diameter is the factor which makes such "peripheral resistance" so significant for the maintenance of the mean arterial blood pressure.

Methods of Determining Blood Viscosity (Hürthle, and Burton-Opitz).—For most purposes the viscosity is measured by comparing the rate of flow through a capillary U-tube (Ostwald's *viscosimeter*) of hirudinized or heparinized blood on the one hand and distilled water on the other. Other anticoagulants alter the values obtained. An average determination shows blood (human) to be approximately five times as "thick" as water.

Factors Affecting Blood Viscosity.—1. Red cells: (a) Numbers—*e. g.*, polycythemias; (b) size and shape—*e. g.*, the viscosity increase in venous and, especially, asphyxial blood is due to swelling of the red cells under the influence of the acidosis.

2. White cells: In leukemias.

3. Plasma proteins: (a) Viscosity is lessened in hydremic plethora (hypervolemia without cell increase); (b) viscosity is increased in acute fevers, secondarily to the water balance disturbance (Barbour); (c) viscosity is increased in all anhydremias (hypovolemias).

4. Normal variations: (a) Age—viscosity increases gradually in first ten years of life and very slightly with increasing age; (b) sex—viscosity is slightly lower in females; (c) minor variations occur in relation to exercise, meals, etc.

The Formed Elements of the Blood.—The blood platelets normally number some 250,000–400,000 per cubic millimeter although none of the numerous methods for their enumeration is free from technical objections. A diminished platelet count (*thrombopenia*) occurs, idiopathically, in purpura hemorrhagica, and, secondarily, in destructive diseases of the bone marrow (*e. g.*, pernicious anemia, acute myelitis—toxic or septic—*x*-rays, etc.). Splenectomy causes a temporary rise in the numbers of circulating platelets suggesting that the spleen is an important organ in platelet destruction. Raised platelet counts occur in myeloid leukemia and in Hodgkin's disease. We have made dark field observations of living bone marrow cells which showed that the megakaryocytes can form "excrescences" closely resembling those of platelets in clotting blood (see *blood coagulation*). We have identified these "excrescences" with the "buds" described by J. H. Wright in Romanowsky (=eosin-methylene blue-azur) stained sections. We believe that there is nothing "specific" about the azurophil granulation, which in platelets may resemble nuclear (chromatin) material, although it is undoubtedly different microchemically and is not now believed to have a nuclear origin. The origin of the platelet as a broken off pseudopod of the megakaryocyte is called into question by these

observations since similarity of behavior is a poor criterion of identity and the difference between an excrescence and a true cell fragment can readily be appreciated by the dark field technic as applied to living cells. A tiny nucleated cell in mammalian embryonic blood was found, in our dark field studies, to have a similar behavior to platelets and megakaryocytes.

(The importance of the phospholipoids of the blood platelets will be discussed under Blood Coagulation.)

The **leukocytes** number, normally, some 7000 ± 3000 per cubic millimeter, according to the approved methods of hemacytometry.

A differential count of the white cell types usually found in the peripheral blood averages: Neutrophil polymorphonuclears, 65 per cent; eosinophils, 5 per cent; basophils (mast-cells), 0.5 per cent; lymphocytes, 25 per cent; and monocytes (large mononuclears or hyaline cells), 5 per cent. Abnormally, marrow, lymph, and histiocytic cells may get into the circulation.

A diminished total leukocyte count (*leukopenia*) occurs *pari passu* with the red cell decrease in destructive bone marrow diseases (especially pernicious anemia) and also in some cases of starvation and malnutrition (*e. g.*, the cachexia caused by cancerous stricture of the esophagus). A leukopenia in acute sepsis is an extremely grave sign; in typhoid fever it is usual.

Increase in one or other cell type, with or without a definite increase in the total leukocyte count (*leukocytosis*), occurs in the following cases:

1. Relative neutrophil granulocytosis (*polymorphonucleosis*):

- (a) Sepsis: Neutrophil increase is a prominent finding in many infectious diseases; the degree of leukocytosis is often a significant index of the success of the "cellular defense mechanism"; the absence of leukocytosis is just as noteworthy in certain infections, *e. g.*, uncomplicated tuberculosis, typhoid, influenza, measles, mumps, malaria, etc.

- (b) Secondary anemias and cachexias; and experimental lowering of blood oxygen by low barometric pressures, carbon monoxide poisoning, etc.

- (c) After experimental injections of dilute hydrochloric acid, cinnamic acid, or of "aleuronat":

2. Relative eosinophilia:

- (a) Many parasitic infestations, especially worm diseases, *e. g.*, ankylostomiasis, trichiniasis, hydatid cyst, etc.;

- (b) A number of skin diseases including pemphigus, eczema, scleroderma, psoriasis, syphilides, urticaria, and, not uncommonly, scarlatina;

- (c) Allergic conditions, such as asthma and other affections believed to be due to hypersensitization to foreign proteins:

3. Relative lymphocytosis:

- (a) In many conditions during infancy and childhood, especially whooping cough, measles, poliomyelitis, rickets, congenital syphilis,

glandular fever, and sometimes in tuberculosis and chronic intestinal infections;

(b) A relative increase in lymphocytes during a leukopenia which affects principally the granular leukocytes is seen in typhoid fever, some protozoal infections, chronic tuberculosis of lymph glands, Hodgkin's disease (occasionally), splenic anemia, and primary anemias, cachexias, pellagra, etc.

(c) Experimentally, various forms of radiation therapy:

4. Relative monocytosis:

(a) Malaria and some other diseases, including Malta fever; sub-acute bacterial endocarditis, convalescence from rheumatic fever, etc.;

(b) Experimental injection of *Bacillus monocytogenes* into rabbits;

(c) Sometimes following the injection of nonspecific proteins and colloidal dyes (due to stimulation of the reticulo-endothelial system).

5. Basophil increase is noted only as an accompaniment of myeloid leukemia.

Myelocytosis, or the appearance of marrow cells in the peripheral blood, may occur during the severe leukocytosis of pneumonia, small-pox, virulent diphtheria, anemias, and certain toxic conditions including uremia, diabetic coma, and exophthalmic goiter. Marrow cells constitute a diagnostic feature of the blood picture in myeloid leukemia.

The leukemias are conditions of such great increase in leukocyte formation that the diseases are frequently believed to be neoplastic (Minot *et al.*). There are two main types: (a) *Myeloid* leukemia—in which all types of bone marrow cell appear in the general circulation, and (b) *lymphatic (lymphadenoid)* leukemia in which the leukocytes are chiefly the small and large lymphocytes.

The Biochemistry of the Leukocytes.—*Specific Granulation.*—Differential staining of granularities in the cytoplasm is an approved method of distinguishing the various leukocytic types. Various modifications of the Romanowsky (eosin-methylene blue-azur) technic have the widest vogue and we may list the methods of Wright, Giemsa, Leishman, May and Grunwald. The *neutrophil* cell is named for its smallish neutrophilic granules; the *basophil* for its larger and more definitely basophilic granules; while the *eosinophil* has conspicuous acidophilic granules. Some of the granular marrow cells are *amphophil*; *i. e.*, containing both basophilic and eosinophilic granules. The *azurophil* granulation which is coarse and scanty in the mature lymphocyte, and finer and more numerous in the monocyte, is of doubtful specificity. Iodophilic and oxidase granules have been described in certain of the granulocytes (leukocytes and their immediate precursors). It may be stated that the earliest precursor cells of the various lines of leukocytic development (myeloblast, lymphoblast, and (?) monoblast), when stained by the Romanowsky methods, have a deep blue hyaline cytoplasm. As differentiation proceeds the cytoplasm becomes paler with the elaboration, firstly, of azurophil granulation (lymphocytes and monocytes) and, secondly, of the specific granulo-

cytic types. Nuclear modifications accompany the cell differentiations and we may allude to the *Arneth count* (and its modifications) in estimating the age of polymorphonuclear leukocytes from the average number of lobulations in the nucleus. We shall not discuss the Golgi bodies.

Living Cells.—The significance of the microchemical appearances must be correlated with data on the living cells, and such have been pursued in recent years by the use of (a) the dark field technic and (b) "vital staining" methods. The dark field (Ferguson, 1930) reveals the existence of cell granules during life and the five classical varieties of leukocyte may be identified by these and other criteria (motility and phagocytic activity). Mitochondria may also be seen with the dark field, but ordinary illumination and vital staining by janus green is the best method of demonstrating these objects.

Phagocytosis.—Lymphocytes are nonphagocytic, but the other varieties of leukocytes are chemiotactically attracted to cocci, yeasts, dead tissue, and other "foreign" materials, and ingest them into their cytoplasm (phagocytosis). The granulocytes are distinguished for their phagocytic activity toward the organisms responsible for acute septic conditions. Their destruction by such organisms results in pus formation (suppuration). The monocytes are also active phagocytes not only for the pyogenic organisms but also for acid-fast bacilli (*e. g.*, leprosy, tuberculosis), fungi, protozoa, etc. They have an especial affinity for dead material and foreign objects (dusts, ova, etc.) and share with the tissue macrophages (large phagocytic cells or histiocytes) the brunt of the reparative processes following tissue injury or infection. There is good evidence from the study of the origins and functions of the blood monocytes and the tissue macrophages (mobile and sessile) to support the claim of Aschoff that they should all be grouped together as the *reticulo-endothelial system*. A rather peculiar kind of phagocytic mechanism occurs in all these cells in connection with the removal of colloidal dyes and various other "sols" (*e. g.*, India ink, collargol, etc.). The taking up of these materials is followed by a vacuole formation in the cytoplasm of the macrophage. In these vacuoles the colloidal particles are aggregated and concentrated, and, if protein in nature, they undergo a slow digestion and finally disappear. The special rôle of the reticulo-endothelial cells in giant-cell formation deserves mention.

The *enzymes* of leukocytes are of some biochemical interest (Opie, Fleischmann). The granulocytes produce a trypsin-like proteolytic ferment (leukoprotease) which acts best in an alkaline or neutral medium. The lymphocytes and monocytes contain a pepsin-like protease (lymphoprotease) which acts only in a feebly acid material. It used to be thought that these ferments were "endocellular" and liberated only during the leukocytic destruction accompanying the inflammatory reaction. It is now believed (Opie) that the proteolytic enzymes may be secreted and normally fail to act because of anti-

enzymes. Jobling and Peterson found antileukoprotease to be associated with unsaturated fatty acids which could be "inactivated" by iodine saturation or heating to 70° C. Fleischmann found lipolytic enzymes in all leukocytes, not only in the lymphocytes and monocytes, as believed heretofore. The inability of leukocytes to digest the acid-fast bacilli (tuberculosis: leprosy) is thought to be associated with specific inhibitory substances in the lipid envelop of these organisms as well as to the inhibitory action of the soaps and fatty acids arising from the action of lipase from the leukocytes themselves. Diastatic enzymes have also been described in the leukocytes, as well as the *oxidases* we have mentioned under Staining Reactions.

The **erythrocytes** are by far the most numerous of the "formed elements" of the blood, numbering some 5,500,000 in man and 5,000,000 in woman (per cubic millimeter of blood). A significant decrease in the red cell count is termed *anemia* (in the specific use of the term) although *oligocythemia* would be better. *Polycythemia* denotes an increased erythrocyte count.

Anemias.—The lowering of the number of circulating red cells is due to a preponderance of destructive influences (causing their removal) over regenerative processes (usually limited, in the adult human, to the bone marrow). The "cell picture" in anemia has been clarified by the study of red cell formation (*erythropoiesis*) in the adult bone marrow, and in the primitive blood islands, the liver, spleen, lymph glands, and bone marrow of the embryo and fetus. It has been shown that the earliest precursor cell is the polyvalent cell which lines the primitive blood sinusoids of the embryo. In contradistinction to leukocyte formation, erythropoiesis always takes place intravascularly (Doan, Cunningham, Sabin). Prior to the appearance of hemoglobin, the mother cell (hemocytoblast) shows a "roset-like" formation with supravital dyes. Hemoglobin gradually replaces the basophilic cytoplasm and the mitochondria lessen in number. The mixed staining reactions of the basophil cytoplasm and eosinophil hemoglobin may be elucidated by observing the living cell. The earliest hemoglobin-containing cells (*megaloblasts*) are large, like the stem cell, and still retain the power of mitotic division whereby smaller daughter cells are formed (*normoblasts*). The term *erythroblast* is appropriate when the nucleus begins to undergo regressive changes which ultimately lead to its disappearance, leaving the mature erythrocyte. Nuclear remnants are rarely seen, *e. g.*, the Cabot "ring bodies" and the so-called "Howell-Jolly bodies." The residual basophil cytoplasm assumes a reticular appearance as the hemoglobin formation increases, and supravital staining with the brilliant cresyl blue brings out the reticulum even after loss of the nucleus. Red cells responding to this test are called *reticulocytes*. Normally, they are rare (less than 2 per cent) in the peripheral circulation but they appear along with nucleated red cells, etc., during the excessive erythrocyte regeneration accompanying anemias (except the aplastic type).

The erythrocyte count is subject to a certain measure of physiologic control by mobilization of the red cells stored in the spleen and bone marrow. The oxygen demand of the body is the chief factor controlling erythropoiesis and explains not only the recovery from anemias but also certain polycythemias such as those occurring in acclimatization to high altitudes or to congenital anomalies of the circulation, etc. We shall not discuss the problems connected with the life-span (probably eighteen to fifty days) or the physiologic removal of effete red cells, in which the reticulocytes of spleen, liver, and bone marrow play the most important part.

(The biochemistry of the red cell is largely that of its hemoglobin pigment and of its ion equilibria, which are dealt with in other chapters.)

The erythrocyte protein is specific in a number of immune reactions such as the precipitin and complement-deviation tests by which the species from which a blood stain originated may be identified with considerable certainty. "*Complement*" (*alexine*) is a normal plasma constituent which has recently been identified with prothrombin. It is an intermediary in the immune reactions of agglutination and hemolysis, etc., the other factor being the specific agglutinin or hemolysin respectively. The value of agglutination tests in separating the four main "blood groups" and so adding to the safety of human blood transfusions, is established. It safeguards against the risk of red blood corpuscle agglutination (and subsequent hemolysis) by intraspecies differences in the agglutinins (*iso-agglutinins*). The bloods of foreign species (*hetero-agglutinins*) are usually "incompatible." Leaving these topics of immunological interest for the simpler phenomena which fall more strictly into the biochemical field, we pass on to experimental hemolysis.

Hemolysis.—In all the higher forms of animal life the hemoglobin pigment is contained in the red blood corpuscles and its respiratory functions are greatly enhanced by the resulting physical conditions (Barcroft). Although erythrocytes are not normally destroyed by hemolysis in the body even in most anemias, and the plasma affords a considerable degree of protection against hemolytic influences, yet hemolysis can occur *in vivo* as well as *in vitro* and it is therefore an important subject for biochemical investigation. *Hemolysis* consists of the destruction of the red cells and consequent liberation of hemoglobin into the surrounding fluid, the process frequently being termed "*taking of the blood.*" The causal factors in simple *in vitro* hemolysis fall into two groups: (1) An osmotic imbalance between the red cell contents and the surrounding fluid, and (2) specific changes in the colloids of the cell "membrane" or envelop, or perhaps of the "gel" stroma, resulting in alteration of "permeability," or of "colloidal relations."

Osmotic hemolysis, like other cell "plasmolysis" is due to water passing into the cell causing swelling and ultimate dissolution. The

classical explanation is based upon the theory that there is a surface membrane of selective permeability restraining the passage of dissolved substances but readily permitting the passage of water and so creating an "osmotic pressure" which may be looked upon as the kinetic force which transfers solvent from the purer solvent (weaker solution) to the more "diluted" solvent (stronger solution) of lower total kinetic energy. The passage of water into a cell is termed *endosmosis* or *osmotic imbibition*. Hanzlik has recently called attention to another viewpoint which is based upon the behavior of "gel" colloids. The phase relations of such gels are under the direct control of electrolytes and other dissolved substances, and these determine the water content and degree of swelling of such gels. If the red cell colloid is in the "gel" state, and there are many arguments, based on its biconcave shape, the absence of brownian movement (or indeed of any particulate matter) in its interior, etc., then Hanzlik's views may hold. The alternative theory believes the erythrocyte to consist of a little vesicle surrounded by an envelop or membrane. Whatever the ultimate mechanism, there is no doubt that the red cell is very subject to volume changes in response to osmotic and other factors. It can swell to a sphere and enlarge considerably, before actually disintegrating, thus taking care of the normal range of osmotic variations in the plasma at different times. Normal volume fluctuations should be taken into account in evaluating red blood corpuscle diameter counts.

Laking of the blood by alternate freezing and thawing is probably due to local hypotonicity in the region of the melting ice crystals.

Individual red cells vary considerably in their resistance to osmotic hemolysis which somewhat complicates the experiment of inducing hemolysis by means of serial dilutions of hypotonic sodium chloride solution. This procedure is used clinically under the designation of the *red blood corpuscle fragility test* (Rous). On the average, visible hemolysis commences in 0.45 per cent NaCl and is complete in 0.35 per cent solution.

In hemolytic jaundice, both inherited and acquired, the red cell fragility shows a definite increase so that salt solutions nearer isotonicity (0.9 per cent NaCl in man) will produce hemolysis. In anemias the fragility is often lessened.

The measurement (by the hematocrit) of red cell volumes in a series of salt concentrations is one of the most practical methods of estimating the physiologic "isotonicity" of solutions, but in practice it is complicated by such technical difficulties as a trace of hemolysis, doubt as to standard rate of centrifugalization, etc.

Hemolysis, believed to be due to a primary change in the red cell "membrane," or (Hanzlik) in the colloidal "gel" of the cell as a whole, includes:

1. Mechanical hemolysis—shaking *in vitro* (Meltzer, Fenn), or tapping an exposed artery in an animal experiment (Ewald).

2. Radiation hemolysis—sunlight, ultraviolet rays, x -rays, radium (β -rays), etc., *in vitro*.

3. Thermal hemolysis—*in vitro* temperatures over 50° C.

4. Hemolysis by lowering of surface tension—bile salts, soaps, saponin, etc. Hemolysis by narcotics, such as alcohol, ether, chloroform, etc., probably falls into this class.

5. Hemolysis by pH changes—is best explained on Hanzlik's theory of colloidal relations.

6. Hemolysis by hemotoxins (*e. g.*, snake and spider venoms, anaerobic sporing bacilli of tetanus and gas gangrene, hemolytic streptococci, etc.) and the action of *specific* hemolysins (possibly).

The effects of *in vivo* hemolysis are hemoglobinemia and hemoglobinuria with the attendant symptoms of chills, fever, malaise, cyanosis, and "shock," etc. *Paroxysmal hemoglobinuria* is a special clinical type sometimes related to syphilis or malaria. Strenuous exercise or cold often determines the onset of an attack.

Plasma and Serum Proteins.—In the chapter on Blood Coagulation is cited the view (Pickering, 1928) that the plasma proteins must be looked upon as a closely knit complex of interrelated substances. Several data suggest that the plasma (or serum) can function as a physicochemical unit; others point to a great variability in the amount and composition of the various "fractions" obtainable therefrom by artificial means. Hardy (1905) showed that serum behaved like a uniform medium in its conduction of the electrical current. More recent methods of electrodialysis, on the other hand, can separate several plasma (or serum) fractions. The classical method (Dennis, 1856; Hammarsten, 1880, *et al.*) of fractionating plasma proteins is by differential salting with serial concentrations of neutral salts, especially ammonium sulphate, magnesium sulphate, sodium sulphate, and sodium chloride. Howe (1921) and Davide (1925) have paid strict attention to the exact strengths of salt solutions required to precipitate the various fractions. Fractional heat coagulation (Halliburton, Huiscamp, Iscovesco, *et al.*) also points to a complex composition of the plasma (or serum) protein. The correspondence between the thermal coagulations of whole plasma and the coagulation temperatures of salted-out fractions is only approximate. Hardy's physicochemical studies led him to believe that the processes of fractionation did not follow the phase rule but the yield of each fraction was unduly influenced by the amount of precipitate already formed. Sørensen (1925) extended our physicochemical data and concluded that there were probably three interrelated serum proteins (? polypeptides): Salt (ammonium sulfate) precipitations yielded varying mixtures of the three primary complexes. Svedberg (1928) found only two fractions by the use of the ultracentrifuge. Mutzenbecker (1933), with the same technic, separated an indefinite number of fractions from serum and citrated plasma, and believed that the result was determined by the size of "molecular aggregates" which could be modified by numerous

casual factors such as standing, or dilution with water (even the ingestion of water before taking the blood sample). In all fractions obtained with the ultracentrifuge, both "albumin" and "globulin" could be demonstrated. Block (1934), using Vickery and Leavenworth's methods for the determination of basic amino acids, has demonstrated the uniformity of the lysine: arginine ratio in sera from different animals, and human plasma both normally and in nephrosis (even with a fall in the albumin: globulin ratio from 1.6 to 0.15), although the data on salted-out fractions prove that differences between these protein fractions occur in respect of their lysine content which varies between 4.3 per cent for the least soluble and 39.6 per cent for the most soluble fraction, the arginine remaining constant at 5.7 per cent. Block concludes: "Mammalian *orosin* (= total coagulable serum protein) is composed of two or more unstable coprecipitation systems in mutual equilibrium." Chick (1914) found that the differences between "euglobulin" and "pseudoglobulin" could be attributed to the lipid (phosphatide) content of the former. Cekada's data on "prothrombin" (see Blood Coagulation) are in accordance with these views. The question of "impurities" such as lipoids, salts, and incidentally adsorbed substances, as well as the difficulty of obtaining sharp separations, demand a critical attitude toward the claims of any protein fraction to be considered as a chemical individual. A further criticism concerns the possibility of denaturation which is inseparable from all the foregoing methods of fractionation. The modern viewpoint accepts, with these reservations, the classical conceptions of the chemistry of the plasma proteins which are presented (briefly) in the following paragraphs.

The plasma proteins constitute nearly 7 per cent of the plasma weight, some 0.3 Gm. as fibrinogen, 1.7 Gm. as serum globulin, and 4.5 Gm. as serum albumin.

The plasma globulins constitute a group of protein fractions which may be precipitated, sometimes incompletely, by the following methods:

1. Fractional heat coagulation—below 75° C.
2. Differential salting out of neutral solutions by (a) half-saturation with $(\text{NH}_4)_2\text{SO}_4$ or (b) complete saturation with MgSO_4 .
3. Dialysis, especially electrodialysis, since at least a trace of salts is necessary to preserve the colloidal solution.
4. Weak acids, including a stream of CO_2 .

Careful salt fractionation, spontaneous coagulation, and dialysis give grounds for believing the plasma globulin to be a complex of two (possibly three) constituents.

Fibrinogen can be distinguished by (a) lower heat coagulation (56° C.); (b) complete salting-out by saturation with sodium chloride, and weaker concentrations of other salts (e. g., 20 per cent $(\text{NH}_4)_2\text{SO}_4$); and (c) spontaneous clotting under the appropriate conditions which are discussed in the section on blood coagulation.

Serum globulin (paraglobulin) does not enter into the essential formation of fibrin clots, but remains in solution after the foregoing procedures for separating out the fibrinogen. Two varieties are usually described: *euglobulin* separated by one third (28–33 per cent) saturation with ammonium sulfate, or by simple dialysis; *pseudoglobulin* requires half-saturation (34–50 per cent) with $(\text{NH}_4)_2\text{SO}_4$, or electrodialysis. It is difficult to free it from albumin.

We have alluded to Chick's dialysis data which point to a phosphatide union (lipoprotein conjugation) as the chief distinguishing characteristic of euglobulin. Cekada's experiments with prothrombin so closely resemble Chick's findings that they strengthen the view that the serum globulins play a rôle in clotting by supplying the prothrombin which is the precursor of the coagulant in thrombin clotting. Should "complement" (alexine) turn out to be an identical or related body (Fuchs), the functions of the plasma globulins must be extended to include a large range of immunity reactions.

The *serum albumins* constitute an ill-defined group of fractions which are precipitated as a unit on full saturation with $(\text{NH}_4)_2\text{SO}_4$, and crystallize out together in the purest forms which have been prepared. Fractional heat coagulation (Halliburton, *et al.*) gives an indication of several components flocculating at 73° C., 77° C., and 84° C., respectively.

Molecular Weight of Plasma Proteins.—Svedberg (1928) has used an ultracentrifuge, developing well over 100,000 times the force of gravitation, in computing the molecular weight of proteins. Whether he is dealing with isolated molecules or merely colloidal aggregates (micellae) it is impossible to say. Serum albumins give a value of 68,000 which is close to that for hemoglobin. Serum globulins average about 103,800. Adair and Robinson (1930) confirmed the results for albumin, but placed the globulin at a higher value, averaging 175,000. We have noted (p. 409) the discrepancy between osmotic equivalents calculated from these values and those actually obtained by osmometry (Govaerts).

The albumin:globulin ratio (Moore and van Slyke, 1929) has interested clinicians because of the variations demonstrated in certain diseases, particularly *nephrosis*. A drop from the normal ratio of 1.5 or 2 to 0.5 or under, is not uncommon in this disease, and is correlated with a loss of more albumin than globulin in the urine, which might be accounted for on the basis of the smaller molecular weight of the albumin fraction. In view of the possibility of basic changes in the plasma ion balances, etc., which might modify the conditions of salt precipitation of the plasma proteins, and the fundamental constancy of the basic amino-acid ratios in nephrosis (Block), the albumin:globulin ratio would seem to be shrouded in a considerable degree of empiricism. Studies of the protein disturbances without simultaneous data on salt and fluid balance are valueless (Peters).

Plasmapheresis.—This consists in removing the blood from an experimental animal (dog) and reinjecting the washed corpuscles in Ringer-Locke suspension. In this way the plasma proteins may be depleted to any desired extent. The method was first used by Kerr, Hurwitz, and Whipple (1918-'20) to study the regeneration of the plasma proteins. Fibrinogen was restored to normal levels in about six hours, provided the liver function was intact. The remaining proteins regained some 1 per cent in twenty-four hours but restoration to normal required one to two weeks. Serum globulin regeneration was completed before that of serum albumin. Reduction of the plasma proteins from their normal level of 6.5–8.5 per cent to 1 per cent resulted in "shock." Leiter (1928-'31) made use of plasmapheresis to clarify the understanding of edema formation. This occurred with plasma protein levels below 3 per cent, and could be explained as due to a lowering of the "osmotic resistance" of the plasma colloids to filtration *via* the capillaries under the influence of the hydrostatic (blood) pressure. It fits in with the Starling theory of capillary permeability (1895-'96), and the recent data of Krogh, Landis, White, *et al.*

Malnutritional edema (Maver, 1932), such as occurs in famines, is accompanied by decrease in plasma protein, and has been experimentally produced by low-protein diets in rats and dogs (Kohman, 1920).

The edema of nephrosis has received much attention in the light of these views and Epstein (1917), Frisch, Mendel, and Peters (1929), Shelbourne and Egloff (1931), Weech, Snelling, and Goettsch (1933), and Leiter (1931) have emphasized the loss of plasma protein and the lowered colloid osmotic pressure (100 mm. of water—Krogh) in this disease. The "edema level" in man corresponds to a serum protein level of about 5 Gm. per cent, but depends to some extent on the albumin: globulin ratio, an "albumin level" of about 2 Gm. per cent usually being recognized. The study of the protein content of the edema fluid is important in explaining and differentiating edema formations. A too simple physicochemical explanation is to be deprecated in the light of other accompanying disturbances such as a lowered basal metabolic rate and a not uncommon rise in blood cholesterol. Salt imbalance is a very common concomitant and chloride retention may be an integral part of the picture, although it is not always demonstrable.

Other plasma proteins have been described from time to time but satisfactory substantiation of their identity and significance has not been forthcoming. The case for a nucleoprotein (Halliburton, Wright, *et al.*) would seem to be worthy of notice, were it not for the fact that the biochemical criteria are insufficient to distinguish these data from the positive knowledge we have been accumulating (since Woolridge) on the rôle of phosphatides and lipoproteins in blood coagulation (*q. v.*)

The iso-electric point of the blood proteins is about pH 4.5, so that at the blood pH (7.25) they behave as weak acids, holding in combination a considerable amount of base (equivalent, according to van Slyke, to 17 cc. of $N/1$ $NaOH$ per 70 Gm. of plasma protein, contained in 1 liter). The base is an important part of the "alkali reserve" (van Slyke) which is available for forming bicarbonates with CO_2 (H_2CO_3), the proteins thus functioning as buffer substances and aiding in the transport of CO_2 .

BLOOD COAGULATION

The undisturbed blood plasma circulates in the vascular system of the healthy body without evincing any tendency to clot. Clot formation consists in the separation from whole blood, cell-free plasma and related fluids, and "purified" plasma fractions, of a protein-containing material originally named *fibrin* by Chaptal in 1795. Fibrin evinces many criteria of chemical individuality, but there is considerable evidence to show that the clot is uniform neither in amount nor in composition. It is particularly influenced, in ways which are not altogether clear, by the incorporation of other proteins present, so that we may refer, at least to the crude material, as α fibrin. Hence we suggest the following as a conservative working definition particularly applicable to mammalian "fibrin": A specific type of protein-containing complex obtainable from blood plasma and related fluids, under appropriate conditions, in the physical state of a colloidal "hydrogel," exhibiting (typically) a double-refracting quasicrystalline structure. It follows that the term "fibrinogen" be acceptable for: All constituents of plasma and (in reasonable fairness) of other body materials which contain protein substances that actually enter into the essential formation of fibrin clots. It is misleading to accept the classical definition, *viz.*, that fibrinogen is a globulin coagulable by thrombin, since chemistry cannot admit the term "globulin" to higher status than a provisional title for a group of crude proteins, and "thrombin" is an equally empirical term for an artificially prepared blood product and the last word is still to be said about its composition and the conditions which govern its action. Studies with tissue coagulants prove that thrombin is not the only material known to produce typical fibrin formation.

Clotting Factors.—Wooldridge (1893) was the first to point out that clotting factors fall naturally into two groups. Firstly, there are agencies which do not seem to be directly concerned with the composition of the clot but rather with the physical conditions under which coagulation may take place. When blood is removed from the body it is at once exposed to an altered environment which disturbs its physical state. For instance, there are temperature changes, and also disturbance in gas and ion equilibria, the H -ion and Ca -ion equilibria being especially important. The chief physical factor, however, from the viewpoint of coagulation, is the surface-tension lowering effect of "wetting" or contact with foreign objects, nonspecific colloids (*e. g.*,

gelatin), and the like. Secondly, there are factors taking part in a series of colloidal interactions which we may tentatively label "chemical." These we shall speak of as *fibrin factors*. An extremely critical attitude is called for in evaluating the properties of the components of the clotting process after they have been isolated and "purified." Such procedures originated with Alexander Schmidt (1861-1895) and Olof Hammarsten (1875-1900) and have been used extensively, with most conspicuous success in the hands of W. H. Howell and his co-workers (1910, etc.).

In the first place it may be inquired whether the "drastic disintegration" (Pickering) of the colloidal complexes of the plasma does not rob the artificially separated fractions of certain significant properties which the undisturbed plasma possesses in virtue of just that normally integrated complexity. This aspect of the problem is by no means unapproachable experimentally; neither does it detract from the proved advances made by studying the artificially isolated factors. Secondly, we must control our tests to insure a corresponding degree of purity in all the reagents by which we test the coagulation reactions. It is useless, for instance, to test out a highly purified thrombin on crude plasma or a fibrinogen of unknown lipoid content (Ferguson, 1934). There can be no doubt that most of the confusion which has existed concerning the rôle of the isolated fibrin factors has been due to the fact that different workers have experimented with materials of varying purity and under poorly defined test conditions. We would list the following test requirements in establishing the rôle of each clotting factor. The first requirement for investigating the part played by any single factor is to be able to test accurately and exclusively for its presence. If each factor could be isolated in chemically pure form or identified by means of specific chemical reactions (applicable under the experimental conditions) the problem would resemble many a similar one in biochemistry. This is not the case with the study of blood coagulation. The only reliable tests involve the behavior of each factor in the actual clotting process, where it operates in conjunction with a multiplicity of interrelated factors.

In making our tests, therefore, we must also be certain that these other variables are under strict control. This essential requirement is by no means easily fulfilled and failure to insure it has resulted in a host of unreliable data. A further requirement is the narrow definition of the conditions of operation of each clotting factor.

Fibrin Factors.

A. In plasma.

1. Plasma fibrinogen
2. Prothrombin (thrombogen, serozyme)
3. Antithrombin
4. Antiprothrombin (heparin)
5. Plasma lipoids
6. Calcium

B. *In platelets and damaged cells.*

7. Tissue lipoids (cephalin; cytozyme; thrombokinase; thromboplastic substance)

8. Tissue coagulant (coagulin: tissue fibrinogen)

C. *In clotted blood.*

9. Fibrin

10. Thrombin (thrombase: fibrin ferment)

11. Metathrombin

The foregoing are to be looked upon as properties of empirically prepared materials rather than as established chemical entities. The isolated "fibrin factors" must be considered, not merely in relation to their specific properties and *in vitro* reactions, but also for their probable significance (as attested by good experimental evidence) in the naturally occurring clot processes. The all-important question in the case of the cell factors is how these are made available in the plasma.

Fibrinogen (Schmidt, 1861; Hammarsten, 1877). This plasma fraction has all the characteristics of the so-called "globulin" type of protein, and, in addition, it is the essential chemical factor in fibrin formation. It is unusually easy to precipitate (by no means in a pure state) by such agencies as neutral salts, half-saturation with sodium chloride usually being employed. Its solutions are heat coagulable at about 56° C. in the presence of neutral salts. After its removal by salt precipitation or heat coagulation, the residual proteins of the plasma are unable to form fibrin upon the addition of all the other necessary factors. The criteria of purity of fibrinogen solutions have received inadequate consideration from most investigators. It is necessary to determine how its rôle in clotting is influenced by the following known "impurities": (1) Salts, especially of calcium, (2) lipoids (cephalin), (3) prothrombin, (4) serum globulin (the "paraglobulin" of older workers)—this last is the globulin fraction which remains uncoagulated even when the best preparations of fibrinogen are used.

Clotting Reactions of Fibrinogen Solutions.—1. No spontaneous clot, even at 38° C. for many hours (Mills), if free from thrombin or active tissue coagulant.

2. No clot with (1) calcium alone, or (2) calcium + added cephalin (Mills), if free from prothrombin.

3. Ready clot with (1) thrombin (extracted from clot), even in the absence of ionized calcium (Hammarsten, Pekelharing, Howell), (2) "activated" prothrombin (*i. e.*, prothrombin + calcium + cephalin), and (3) tissue coagulant + calcium.

Occurrence and formation of fibrinogen.—There is enough fibrinogen in the postembryonic blood plasma of normal vertebrates, and of nearly all abnormal bloods, including hemophilia (Wöhlisch, 1923), to cause a solid clotting of the whole blood. In the chick embryo (Sabin, 1920), a primitive plasma commences to form in the hemo-

poietic "Anlagen" (blood-cell islands) on the second day of incubation: An "albumin"-type protein fraction is demonstrable on the eleventh day; but coagulability is not evident until a day or two later when a "globulin"-type fraction, including both fibrinogen and prothrombin, becomes demonstrable. While there is a strong *a priori* presumption, therefore, that fibrinogen is formed throughout life in the blood-forming organs generally, the experimental evidence points especially to the liver (Doyon, Whipple, *et al.*). After extirpation of the liver, or an Eck-fistula operation, the plasma fibrinogen of a dog falls off rapidly and is not regenerated by protein administration. When the blood proteins are experimentally reduced as in a *plasmapheresis* experiment, the fibrinogen is quickly restored, especially by protein feeding and blood transfusion, whereas the other plasma proteins are replaced very slowly (days or weeks). The demonstration of fibrinogen in liver perfusates and the marked reduction in blood fibrinogen during certain pathologic and experimental conditions of gross liver injury again point to the liver as the chief source of fibrinogen in post-natal life. Extraction and perfusion experiments on bone marrow (Drinker, *et al.*) have given suggestive but inconclusive results.

Prothrombin (Schmidt, Pekelharing, 1891).—This protein fraction is associated (probably exclusively) with the "pseudoglobulin" remaining in the plasma after the removal of fibrinogen. It can be isolated in a state of questionable purity by differential salting, half-saturation with ammonium sulphate usually being employed. Howell (1912) introduced a special "acetone method" which has been criticized, especially by Mills (1927). It is still a common error to look upon prothrombin as originating in platelets and cells (see later), but Mills' (1927) refutation is final. It is abundantly present in cell-free plasma. Fuchs (1933) has recently suggested a close relationship between prothrombin and the *complement* (alexine) of the immunological defense mechanisms. The most important "impurities" in prothrombin preparations are (1) salts, (2) lipoids (cephalin), and perhaps (3) tissue coagulant.

Clotting Reactions of Prothrombin Solutions.—1. No spontaneous clot, even when warmed (38° C.) with added calcium and cephalin, if free from fibrinogen.

2. No clot with fibrinogen unless calcium (Pekelharing, Howell) and also cephalin (Bordet, Mills, Ferguson) be present.

3. Can be activated by chloroform, carbon tetrachloride, and other *in vitro* treatment (Cekada, 1926), but it has not been commented upon that mobilization of a cephalin "impurity" may be involved.

4. Heparin (antiprothrombin) hinders the "activation" of prothrombin by calcium ions, unless an excess of cephalin or tissue coagulant be present.

5. Activated prothrombin (see thrombin) cannot act on fibrinogen if a sufficient quantity of antithrombin be present.

Calcium.—Although foreshadowed by O. Hammarsten (1875-'77),

J. R. Green (1887), and Ringer and Sainsbury (1890), Arthus and Pagès (1890) are justly credited with demonstrating conclusively that calcium salts are essential for blood coagulation. They added to blood *in vitro* oxalates, 0.1 per cent, fluorides, 0.15 per cent, and alkaline soaps, 0.5 per cent and found that the blood remained fluid for several days. The subsequent addition of calcium chloride solution restored clotting in a few minutes. C. A. Pekelharing (1891) was the first to use citrates, and Sabbatini (1900-'03) employed citrates and a number of other salts to establish the contention that the ionized calcium was the all-important form in coagulation. Our knowledge of the mechanism of action and the practical application to blood transfusions of these "decalcifying" anticoagulants has been extended by Mellanby (1908), Lewisohn (1915, 1924), Salant and Wise (1916), Vines (1921), *et al.* Much remains to be cleared up regarding the exact rôle of calcium in clotting, but the following data are accepted: (1) Calcium ions are essential for the "activation" of prothrombin (Pekelharing, *et al.*); (2) also for the action of tissue coagulant on fibrinogen (Mills); and (3) ionized calcium is not necessary for the interaction of thrombin and fibrinogen (Hammarsten, Pekelharing, Howell). We believe that the physical rôle of calcium ions is primarily to control the "osmotic disintegration" of the blood platelets (Ferguson, 1934) and is in some way connected with the lipid: water phase relations (Clowes, 1913, 1916). The possibility of a chemical rôle not only of "free" calcium but also of calcium in specific combination with the lipoids and proteins (Mills) needs further investigation, especially in the light of recent data by de Waele (1926), Loucks (1926), and Rabinovich (1926).

The Lipoid (Cephalin) Factor.—Far in advance of his time, L. C. Wooldridge (1893) contributed many experimental data to the coagulation problem which have not received the recognition they truly deserve. He even isolated a phosphatide (which he termed "lecithin" according to Strecker's nomenclature in use at the time). Among his source materials we may note tissue extracts, the precipitate from cooled (cell-free) plasma, and fibrinogen preparations (Hammarsten's method). His experiments foreshadowed much of our modern knowledge concerning the rôle of lipoids in blood clotting. Howell (1912) and his pupil McLean (1916) proved that the so-called "thromboplastic factor" (Schmidt, Nolf, *et al.*) or "thrombokinas" (Fuld and Spiro; Morawitz) in brain and thymus extracts was, in all probability, the phosphatide *cephalin*. Zak (1912) confirmed Howell, and so did Bordet and Delange (1912, 1913), using chiefly platelets and muscle, although they retained the less accurate term "lecithin," being subsequently supported by Zunz and la Barre (1921), who were not convinced of the exact identity of the phosphatide.

The earlier English work (Halliburton, Wright) implicating the *nucleoproteins* has been "ruled out of court" by the more recent findings. Bordet's views are based on the fundamental proposition that

the phosphatide is the direct "activator" of prothrombin (serozym) in the presence of calcium ions. Howell has steadfastly maintained that calcium alone is sufficient for thrombin formation, and that the function of cephalin is to neutralize the inhibitory factors antithrombin and, more particularly, heparin (antiprothrombin) which normally prevent the initiation of the clotting reactions. The careful experiments of Mills and his coworkers lead to the conclusion that cephalin can act in both ways. A distinction must be drawn, according to Mills, between a readily available form of cephalin which is obtainable from tissues and plasma fractions by extraction with cold benzene, and a firmly bound lipoprotein complex which can only be dissociated by prolonged and vigorous "fat"-extraction.

Clotting Reactions of "Free" Cephalin.—1. Bordet found that a drop of cephalin emulsion added to 0.5 cc. of serum (prothrombin) caused fatal intravascular clotting in twenty to thirty seconds after intravenous injection of the fresh mixture into a rabbit. Neither material, alone, had any effect on clotting, even in considerable amounts. Mills and Mathews (1924) confirmed this experiment and showed that the "activated" serum soon lost its potency on standing, but cephalin could reactivate repeatedly for a number of repetitions. They concluded that the prothrombin \rightleftharpoons thrombin transformation is reversible to a large extent but a slow permanent inactivation occurs (see Metathrombin) which is not reversible by cephalin.

2. Cephalin alone, or the lipid extract of tissues in crude form, have no coagulant properties even if calcium be added.

3. Benzene-extracted prothrombin (Mills, 1927) cannot be activated by calcium alone. The best purified prothrombin preparations behave in the same way.

4. We (1934) confirmed these data and stressed also the fact that benzene extraction of the fibrinogen (or an unusually well purified fibrinogen) is necessary to determine whether cephalin is an essential factor in thrombin clotting. Cephalin-free prothrombin (recalcified) will clot a crude fibrinogen preparation but is inactive after benzene extraction of the fibrinogen unless more cephalin or the benzene extract be subsequently added. We believe this finally disposes of Howell's opposition to the view that cephalin does not play a direct (thrombogenic) rôle.

5. Howell's experimentation leaves no doubt that free cephalin can neutralize heparin (antiprothrombin) and also antithrombin. Thus the addition of cephalin emulsion will clot heparin-, hirudin-, and peptone-plasmas.

Tissue Coagulant.—We prefer this noncommittal term to Mills' (Wooldridge's) "tissue fibrinogen," Loeb's "coagulin," or the earlier terms "thromboplastic substance" and "thrombokinasé." Its use should be limited to the factor which we believe (with Mills) to be the "fixed" cephalin or lipoprotein of tissue (including platelet) extracts. Mills found lung tissue to be an excellent source material

Clotting Reactions of Tissue Coagulant (after Mills).—1. Clots fibrinogen, in the presence of calcium ions, even when the possibility of thrombin formation (or action) is excluded (by anticoagulants or by the use of fibrinogen preparations rigidly controlled for freedom from prothrombin). Nakamura's (1931) dark field observations prove the fibrin formed by the action of tissue coagulant to be identical with that resulting from the use of pure thrombin (activated prothrombin).

2. Additional "free" cephalin increases the coagulant property of the tissue extract.

3. Antithrombin and antiprothrombin are powerfully antagonized by benzene-extracted tissue coagulant; but prothrombin is not activated in the absence of "free" cephalin.

4. After complete lipoid extraction the tissue-extract residue has the reactions of a globulin protein and is strongly anticoagulant, but the addition of the lipoid extract or pure cephalin restores the coagulant property.

The presence of the protein moiety renders tissue coagulant labile to heat (over 60° C.). After boiling it has the properties of cephalin alone, which is heat stable.

Antithrombin.—The observations of Schmidt-Mulheim (1880) and Fano (1880) that the intravenous injection of a nonfatal dose of "peptone" led to a strong inhibition of clotting, not overcome by the addition of "thrombin," led Schmidt to postulate the appearance of an "antithrombin" (or thrombin-neutralizing substance) under these experimental conditions. Wooldridge (1893) noted that his tissue coagulants, when injected intravenously, either caused a fatal intravascular clotting ("positive phase") or a mild degree of thrombosis in the deep circulation (particularly in the portal vein) with a subsequent "negative phase" or incoagulability of the rest of the blood. Added tissue extracts ("tissue fibrinogen") overcame this inhibition, both *in vivo* and *in vitro*. He also noted that large quantities of peptone could hinder clotting *in vitro* if the plasma were obtained from an animal previously starved. The low blood lipoids after a starvation period, and the high values in portal venous blood are important (modern) data in the interpretation of Wooldridge's experiments. Howell demonstrated the presence of small amounts of antithrombin in normal plasma and he confirmed the removal of its influence by means of the cephalin fraction of tissue extracts. Mills has extended the data on antithrombin neutralization by tissue extracts, and is especially to be credited with the discovery of the anticoagulant properties of the protein (globulin) moiety of tissue coagulant ("tissue fibrinogen").

Clotting Reactions of Antithrombin.—(1) It prevents thrombin clotting; (2) it does not prevent tissue coagulant clotting; (3) it is neutralized by (a) free cephalin, and (b) combined cephalin (tissue coagulant); (4) its anticoagulant properties are lost after heating to 70° C.

Types of Antithrombin.—(1) Plasma antithrombin (Howell), normally present in small amounts, but (2) greatly increased in "peptone

shock," (3) hirudin or leech extract (Haykraft; Gratia) and related substances from the tick (Sabbatini) and hookworm (Loeb and Smith) (hirudin is thermostable to 88° C.); (4) some snake venoms, especially *in vivo* (Stephens and Myers; Martin; Barratt; Morawitz; Noc, *et al.*); (5) foreign proteins (see Anaphylactic Shock) (Delezenne; Mosso) and the globulin fraction of tissue coagulant (Mills); (6) some vegetable extracts, etc. (Conradi, Pickering).

Sources of Plasma Antithrombin.—The ineffectiveness of peptone after elimination of hepatic activity by the Eck fistula or in other ways, and the anticoagulant properties of liver perfusates strongly point to the liver as the source of circulating antithrombin (Contjean, Gley, Delezenne, Nolf, Popielski, Denny and Minot). It may be significant, however, that autolysis or other disintegrating processes are generally inseparable from the methods of preparing antithrombin. "Physiologic" methods such as perfusion of fresh liver (Menten, 1920), repeated thrombin injections (Rettger, 1909; Mellanby, 1930), or other attempts (bile, bile-salts, secretin, electrical stimulation) to cause the liver to "secrete" antithrombin have failed (Denny and Minot, 1915).

Antiprothrombin (Heparin).—Howell's studies have demonstrated the existence, even in normal plasma, of a substance identical in its behavior with a material obtainable from liver (and therefore called *heparin*) which is an excellent anticoagulant both *in vivo* and *in vitro*. It acts by preventing the formation of thrombin from prothrombin in the otherwise successful activation by calcium (+ cephalin). It does not prevent the action of thrombin (once formed) on fibrinogen. It can be neutralized by cephalin and tissue extract. Heparin has been prepared in pure enough form for chemical analysis by Howell and Holt (1918), Schmitz and Fischer (1933), and Charles and Scott (1933); but unfortunately the different analyses do not entirely agree. It is probably a glycuronic acid derivative.

Thrombin.—Alexander Schmidt (1862) extracted clotted blood with alcohol and prepared a coagulant which he termed *thrombin*, and later *fibrin ferment*. Blood freshly received from a cut vessel directly into alcohol failed to yield an active extract, leading Schmidt to suppose that it existed in blood in the form of a precursor substance (prothrombin) or *zymogen*. Schmidt's early view that the coagulant came from the leukocytes is still not without its influence in maintaining the common error that thrombin is of cellular origin, although the leading authorities agree that the cells merely provide an activating factor (see *cephalin*) which in some way "activates" the prothrombin now known to be a plasma protein. Howell prepared a highly purified thrombin from a saline extract of fibrin clot by repeatedly washing with chloroform. It had the properties of a simple type of protein (or proteose) and was free from cephalin, calcium, and other contaminants. Howell's pupil, Cekada, dialyzed prothrombin solution and fractionated into two types of thrombin, one containing cephalin and

the other lipid-free. On the basis of these lipid-free thrombins, Howell argues against the need for anything save calcium in prothrombin activation. It is surprising to note, however, that Howell records no controls of the cephalin content of the fibrinogen solutions upon which these coagulants were tested, and even Wooldridge was aware of the high phosphatide content of fibrinogen preparations. The previously quoted data of Mills and the Continental workers argue strongly in favor of the view that cephalin as well as calcium is necessary for thrombin formation. The old stumbling block of the *in vivo* ineffectiveness of thrombin has been removed by the experiments of Bordet, Mills and Mathews (*v. supra*) in which cephalin addition immediately prior to injection produced extensive intravascular clotting.

Fibrin.—The solid material which separates out from the serum after coagulation was termed *fibrin* by Chaptal (1795), and Fourcroy (1807) subsequently popularized the term. Schmidt and Hammarsten studied its physical properties and the conditions determining its formation from plasma fibrinogen in "thrombin clotting." Mills and his coworkers have contributed to our knowledge of the fibrin formed by tissue-extract clotting, which, even in the absence of thrombin factors, is apparently the same substance (Nakamura, 1931). Debate centers around the following components of the clot, with reference to the question: Are they essential constituents of fibrin?

1. *Calcium.*—Hammarsten (confirmed by Howell) repeatedly extracted fibrinogen and thrombin solutions with oxalate to remove all *ionized* calcium. Clotting still occurred; hence it has been generally accepted that calcium is not an essential constituent of fibrin, although it is always obtainable, along with other salts, in the ash from incinerated blood clot. The recent work (see *calcium*) tending to show that fibrin formation may be prevented by the removal of calcium by electrodialysis of fibrinogen and its coagulants, perhaps reopens this question.

2. *Cephalin.*—We have reviewed the reasons for believing that cephalin is an essential component of the fibrin factors at some stage in their reactions, but it is still an open question whether the data require us to consider it an essential constituent of fibrin. The fact that it can readily be extracted from the clot is not of much significance in settling this question.

3. *Thrombin.*—The ease with which thrombin may be prepared by a simple salt solution extract of (washed) fibrin has led to the view that it is not an essential constituent but merely an intermediary in the fibrinogen gelation.

4. *Tissue Coagulant.*—Mills thought he had demonstrated an actual entry of the tissue coagulant into the fibrin formation because of the definite increase in the fibrin (dry weight) yield when clotting was promoted by the addition of tissue extracts. The fact that added proteins such as casein, gelatin, and egg albumin also increase the

yield to some extent greatly weakens the argument in favor of the tissue protein being in any way "specific." We must confess that our present knowledge of the chemical differences (if any) between fibrinogen and fibrin is singularly uncertain, and the question of specific union with the coagulants even as an intermediary phenomenon is far from established. We are equally in the dark as to the conditions determining the lability of such hypothetical unions. Some advances have been made in the physicochemical study of fibrin and much may be deduced by analogy from similar colloidal phenomena of the type usually referred to as "sol-gel" transformations. Hekma (1910-'19) in particular, has prepared alkaline ("sol") and acid ("gel") fibrins in which the "sol-gel" transformation may be effected reversibly and at will. Fibrin is peculiar, but not exclusively so, in that the "gel" is microcrystalline or probably quasi- (pseudo-) crystalline. The microscopical needles were first noted by Schimmelbusch (1886) and subsequently studied, especially with the dark field by Stübel (1914, 1920), Howell (1914-1916), Hekma, *et al.* There are minor differences in the microscopic appearance of fibrin from different animals and under different conditions of formation, but there is nothing definite about the physical properties of fibrin to assist us in more than identifying it. We know of no physical or chemical tests for its "purity."

Fibrinolysis.—In addition to Hekma's reversible fibrins, other examples of clot reversal, especially in its incipient stages, are on record (Pickering). Under certain conditions, the fibrin clot undergoes spontaneous resolution or "fibrinolysis." It is, however, not again coagulable (as a rule) suggesting the cause to be a proteolysis of a rather peculiar kind. Clot reversal is an exceptional phenomenon and fibrin must be regarded, in comparison with many other colloids, as a "gel" of a very irreversible kind.

Metathrombin (Morawitz).—The serum from a recent clot has strong thrombic powers but it loses its effectiveness in a matter of minutes (more or less). Prepared thrombin is much more stable. We have alluded to the rôle of cephalin in reactivating the thrombin sources in serum, and noted at the time that the repeated reactivation was limited owing to the gradual conversion of thrombin into a more stable inactive form which Mills and Mathews call *metathrombin*. It may not be quite the same use of the term as Howell's since this author believes the permanent inactivation is usually effected by union of thrombin with antithrombin, a view that is hard to reconcile entirely with the previously quoted facts. Mills' metathrombin can be reactivated by vigorous destructive measures such as extraction with hot strong salt solutions. The significance of metathrombin formation in ordinary clotting should probably be minimized.

The Nature of the Clotting Process.—Having considered in some detail the properties (coagulant and otherwise) of the various fibrin factors, we may inquire what the actual clotting really involves and what prevents its occurrence, normally, in the circulating blood. Blood

coagulation is not a simple nor uniform process but presents variations according to diversities of circumstance including species peculiarities. In so far as a general statement may be deduced from the foregoing data it may be said that the fibrin "gel" (clot) is the result of a specific phase reversal of a plasma protein (fibrinogen) due to a series of chemical (? colloidal) reactions occurring in an orderly sequence as the result of definite physical conditions.

The classical type of blood coagulation, which has been regarded as typical since the subject began to receive serious attention about the middle of the nineteenth century, is seen in the case of blood collected with minimal tissue contact and allowed to clot in a (glass) vessel. The thrombin theory believes the essential feature to be the formation of an intermediary substance, thrombin, from its precursor, prothrombin (thrombogen). Fibrin formation occurs as the result of the interaction of fibrinogen and thrombin. The enzyme analogy (Schmidt) is not now upheld, except by Mellanby (1933). Considerable divergence of opinion as to details has characterized the views of the many authorities in this field of whom special mention may be made of Schmidt, Hammarsten, Pekelharing, Fuld and Spiro, Morawitz, Nolf, Bordet, Hekma, and Howell. The advances made upon the earlier thrombin theory (Schmidt), in which the thrombin was believed to originate from the leukocytes and act like a ferment, may be summarized as follows:

1. Prothrombin is a plasma globulin (Mills), or (?) acid meta-protein (Mellanby).
2. Calcium is essential for the activation of prothrombin.
3. So is a factor of tissue origin, the normal source being the blood platelets. This is now believed to be cephalin. We have reviewed the differences of opinion over the matter of how it acts.
4. Antithrombin is a normal plasma constituent, probably albuminous in nature, tending to prevent the action of thrombin which may arise from any cause.
5. Heparin (antiprothrombin) is another normal plasma constituent of a simpler nature—probably a glycuronic acid derivative—which prevents the formation of thrombin from prothrombin.

According to Howell, these antibodies effectively maintain the normal *in vivo* fluidity of the blood and the lipoid factor operates in neutralizing them. His positive data are well substantiated but exception may be taken to the dogmatism that precludes the ideas (also backed up by experimental facts) that (1) cephalin is an active agent in thrombin formation, and that (2) other factors may be equally important in preventing *in vivo* clot formation.

The second type of blood coagulation is seen upon the addition of tissue extracts to cell-free plasma (even in the presence of antithrombin) or to fibrinogen solutions free from prothrombin. This type of clotting is probably "physiologic" inasmuch as contact with injured tissues is evident in most cases of the arrest of hemorrhage.

L. C. Wooldridge, long before his time, adduced strong evidence that lipoids (lecithins) played an important rôle in this type of clotting. C. A. Mills has performed some of the best controlled experiments in this field. Active tissue coagulants can be prepared from a variety of sources including lung, brain, thymus, muscle, and platelets. We have reviewed the data covering the functions of tissue coagulants in clotting. Mills' theoretical development is ahead of the experimental facts but invokes some definitely constructive ideas. Biochemists are familiar with lipoid-protein conjugations although it is not yet agreed whether these obey the stoichiometric laws or whether they are less specific colloid adsorption phenomena. The chemical rôle of salt ions is not yet elucidated. With these admonitions we may summarize Mills' views as follows:

1. Free cephalin activates prothrombin in just as essential a manner as the third factor, *viz.*, calcium ions. Thrombin is, therefore, a labile prothrombin + calcium + cephalin conjugation.

2. The cephalin is an essential link in the thrombin-fibrinogen interaction, fibrin (or at least its immediate precursor) being fibrinogen + cephalin + calcium + prothrombin.

3. Conjugated cephalin-proteins (Mills refers to these tissue coagulants by Wooldridge's term "tissue fibrinogens") can combine directly with fibrinogen in the presence of calcium to form a fibrin of the constitution: Fibrinogen + calcium + cephalin + tissue protein (globulin).

4. Various proteins (and we must add "etc." to take care of heparin) owe their rôle in clotting to their avidity for cephalin and the stability of the lipoprotein combinations. Thus,

- (a) prothrombin is a globulin of powerful affinity for cephalin but readily loses the lipoid to other claimants such as the antithrombins, heparin, and the nonspecific proteins which Mills believes to be concerned in the formation of "metathrombin."
- (b) Heparin (antiprothrombin) has a considerable affinity for cephalin and can deviate it from its rôle in thrombin formation.
- (c) Antithrombins are the less specific proteins with smaller degrees of affinity for lipoid but diverting it into much more stable combinations.
- (d) Tissue coagulants ("tissue fibrinogens") have firmly combined cephalin which is not deviable by heparin and antithrombins but has residual affinities for combination with fibrinogen.

As cephalin alone does not clot fibrinogen the protein "vehicle" is all-important for coagulation, although it need not be specific (apart from the above considerations) inasmuch as there are no detectable differences between the fibrins produced by thrombin and by the various tissue coagulants. The difficulty of demonstrating the complex

combinations assumed by Mills may be due to the inability of the complexes to withstand the usual extraction and purification technics. It may also be that they are merely intermediaries which do not survive the physical changes accompanying the sol-gel transformation.

A third type of clotting has been stressed by J. W. Pickering and his collaborators. It is seen in the exceptional case of a plasma, *e. g.*, bird's or fish's, which, even after freeing from all corpuscular elements, may be clotted by simple whipping, vigorous shaking, or sufficiently prolonged standing. Delezenne and Nolf laid the foundations of these observations and were led to the conclusion that blood plasma contained all the essential elements for clotting, but that the normal vascular environment precluded the necessary physical factors (especially "wetting") which were necessary for the interaction to take place. Bordet also stressed the "wetting" factor; particularly in the activation of prothrombin (serozyme) by calcium and cephalin (cytozyme). He even postulated a proserozyme needing "wetting" to start the process of activation. Wooldridge probably had an inkling of the true state of affairs when he concluded that the plasma contained abundant "lecithin," but he was very unorthodox (see Löwit) in denying the existence of the blood platelets as a formed element.

Ferguson's view may be regarded as a *cephalin availability theory*, and may be briefly summarized as follows:

1. There is insufficient plasma cephalin to activate the prothrombin in the circulating blood in the presence of the heparin and antithrombins demonstrated by Howell. After a (fat) meal and in the third type of clotting there may occur "the exception that proves the rule."
2. The cephalin so essential for clotting usually comes from the cells and tissues. The blood platelet is peculiarly significant in this respect. In the circulation this "formed element" (Bizzozero) occurs as a small ovoid disk some one fourth to one half the diameter of a red blood cell. On exposing to an abnormal environment, in which the chief factor is probably the wetting factor, the platelet undergoes a peculiar series of morphological alterations the effects of which are to discharge at least a part of its contents into the plasma. Only after this phenomenon does fibrin formation begin. We have demonstrated that calcium ions play an essential rôle in controlling this process, which may be looked upon as a peculiar type of plasmolysis—a phenomenon encountered with many living cells and known to depend upon ion factors and osmotic imbalance. The similarity of the platelet "excrecences" to "myelin figures" may be very significant in view of the known lipid content of platelets. We believe that the expansion of the lipid-rich platelet surface (just as in myelin figure formation) provides a "membrane" peculiarly susceptible to osmotic disturbance. The calcium ion regulation of the lipid: water phase equilibrium (Clowes) explains why water goes *into* the platelet. The rest of the process is an osmotic disruption which discharges the cephalin colloid into the plasma. Other cells and damaged tissues may pro-

vide cephalin and its complexes, if suitable conditions for their mobilization are present.

In *purpura hemorrhagica*, platelets are greatly reduced in numbers. The clot processes are essentially normal (as seen in the normal values for "clotting time") but an insufficient amount of fibrin is formed to give a firm clot.

In *hemophilia* we have noted (in confirmation of Stübel and Howell) that the platelets are unusually well preserved in respect of plasmolysis (osmotic imbibition) but excrecence formation is only slightly retarded. This resembles the condition of the platelets in citrated blood, but as there is no calcium deficiency nor antithrombin (nor heparin) increase in hemophilia we believe that the underlying factor is a quantitative or qualitative deficiency in the platelet cephalin. Perhaps we may even come to regard it as an "inborn error of (phosphatide) metabolism."

J. W. Pickering elaborates another viewpoint which is implicit in the earlier theories of Nolf and perhaps of Bordet. It is to the effect that the normal circulating plasma must be regarded not as a composite of the artificially isolated fractions but as an *integrated protein complex* which maintains a state of dynamic equilibrium the stability of which is insured by an absence of interaction of the components of the clotting mechanism. The *modus operandi* of this stabilization is rather obscure, but the onset of clotting is supposed to be associated with a definite disintegration of the plasma protein complex due to the physical disturbances accompanying withdrawal of the blood from the intact vessels, contact with wettable surfaces, cooling, etc., and the various artificial methods of treatment, including "salting," electro-dialysis, and fractional heat coagulation. Physicochemical studies of the electrolytic conductivity of plasma and serum (Hardy, Sørensen) and the ultimate amino-acid analysis of salted-out proteins (Block) give evidence in favor of the suggestion that the plasma and serum are whole complexes or at least that their component proteins are interdependent and possibly interconvertible. It may be fair, therefore, to call into question the classical assumptions of the separate pre-existence in undisturbed plasma of the various entities we have considered under "fibrin factors." Failing adequate support for these assumptions, such substances must be considered as artefacts or at the best as intermediaries. The latter designation takes cognizance of the practical value of the data derived from the study of their behavior in experimental clotting.

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REFERENCES

Functions of Blood, Lymph, and Tissue Fluids

1. Bernard, C.: *Leçons sur les phénomènes de la vie communs aux animaux et aux végétaux* (1878); Fulton, J. F.: *Selected readings in the history of physiology*, pp. 307-309 (1930).
2. Henderson, L. J.: *Blood. A Study in General Physiology* (1928).

3. Drinker, C. K., and Field, M. E.: *Lymphatics, Lymph, and Tissue Fluid* (1933).
4. Clark, E. R., and Clark, E. L.: *Amer. J. Anat.*, **51**, 49 (1932).

Blood Chemistry

5. Hawk, P. B., and Bergeim, O.: *Practical Physiological Chemistry* (1926).
6. Peters, J. P., and van Slyke, D. D.: *Quantitative Clinical Chemistry* (1931).
7. Folin, O.: *Laboratory Manual of Biological Chemistry* (1925).
8. Beaumont, G. E., and Dodds, E. C.: *Recent Advances in Medicine* (1934).
9. de Wesselow, O. L. V.: *The Chemistry of the Blood in Clinical Medicine* (1925).
10. Myers, V. C.: *Practical Chemical Analysis of Blood* (1924).

Blood Volume

11. Rowntree, L. G., and Brown, G. E.: *Volume of Blood and Plasma in Health and Disease* (1929).
12. Fleischer-Hansen, C. C.: *Skand. Arch. Physiol.*, **59**, 243, 257 (1930).
13. Whipple, G. H., et al.: *Am. J. Physiol.*, **56**, 313, 328, 336 (1921).
14. Erlanger, J.: *Physiol. Rev.*, **1**, 177 (1921).
15. Marriott, W. McK.: *Physiol. Rev.*, **3**, 275 (1923).
16. Griesbach, W.: In *Bethe's Handbuch d. norm. et path. Physiol.*, **6**, 667 (1928).
17. Seyderhelm, R., and Lampe, W.: *Ergebnisse d. inn. Med. u. Kinderh.*, **27**, 245 (1925).
18. Veil, W. M.: *Ergebnisse d. inn. Med. u. Kinderh.*, **23**, 648 (1923).

Blood Viscosity

19. Neuschlosz, S. M.: In *Bethe's Handbuch d. norm. et path. Physiol.*, **6**, 619 (1928).
20. Tigerstedt, R.: *Physiologie des Kreislaufes*, **3**, 12 (1922).
21. Burton-Opitz, R.: *J. Am. Med. Assoc.*, **57**, 353 (1911).
22. Hürthle, R.: *Arch. ges. Physiol.*, **82**, 415 (1900).
23. Determann, H.: *Die Viscosität des menschlichen Blutes* (1910).

Formed Elements

24. Bethe, A., et al.: *Handbuch der normalen und pathologischen Physiologie* (1928).
25. von Schilling, V.: *Das Blutbild und seine klinische Verwendung* (1933).
26. Naegeli, O.: *Blutkrankheiten und Blutdiagnostik* (1923).
27. Cowdry, E. V.: *Special Cytology* (1932).
28. Maximow, A. A.: *Textbook of Histology* (1931).
29. Maximow, A. A.: *Physiol. Rev.*, **4**, 533 (1924).
30. Sabin, F. R.: *Physiol. Rev.*, **2**, 38 (1922).
31. Sabin, F. R.: *Physiol. Rev.*, **8**, 191 (1928).

Reticulo-endothelial System

32. Sacks, B.: *Physiol. Rev.*, **6**, 504 (1926).
33. Jaffé, R. H.: *Physiol. Rev.*, **11**, 277 (1931).

Platelets

34. Wright, J. H.: *J. Morph.*, **21**, No. 2 (1910).
35. Petri, S.: *Acta path. et microbiol. scand.*, **2**, 23, 97, 277, 357 (1925); 432 (1926).
36. Ferguson, J. H.: *Am. J. Physiol.*, **108**, 670 (1934).

Leukocytes

37. Bunting, C. H.: *Physiol. Rev.*, **2**, 505 (1922).
38. Fleischmann, W.: *Ergebnisse Physiol.*, **27**, 1 (1928).
39. Ferguson, J. H.: *Trans. Roy. Soc. (S. Africa)*, **18**, 317 (1930).
40. Arneth, J.: *Die qualitative Blutlehre* (1926).
41. Opie, E. L.: *Physiol. Rev.*, **2**, 552 (1922).
42. Mudd, S., McCutcheon, M., and Lucké, B.: *Physiol. Rev.*, **14**, 210 (1922).

Erythrocytes

43. Wintrobe, M. M.: *Medicine*, 9, 195 (1930).
44. Rous, P.: *Physiol. Rev.*, 3, 75 (1923).
45. Brachmachari, C.: *Studies in Haemolysis* (1913).
46. Barcroft, J. F.: *Harvey Lectures*, 17, 146 (1921).
47. Barcroft, J. F.: *The Respiratory Function of the Blood* (1928).
48. Jacobs, M. H.: *Harvey Lectures*, 22, 146 (1926).
49. Ponder, E.: *The Erythrocyte and the Action of the Simple Haemolysins* (1924).
50. Ponder, E.: *The Mammalian Red Cell and the Properties of Hemolytic Systems*: in R. Chambers' "*Protoplasma Monographien*," 6 (1934).
51. Hamburger, H. J.: *Osmotischer Druck und Ionenlehre* (1902).
52. Hamburger, H. J.: *Ergebnisse Physiol.* I Abt., 23, 77 (1924).

Plasma Proteins

53. Pickering, J. W.: *The Blood Plasma in Health and Disease* (1928).
54. Howe, P. E.: *J. Biol. Chem.*, 49, 93 (1921).
55. Howe, P. E.: *Physiol. Rev.*, 5, 439 (1925).
56. Davide, H.: *Acta Med. Scand.*, (1925).
57. Sørensen, S. P.: *Proteins* (1925).
58. Svedberg, T., and Sjögren, B.: *J. Am. Chem. Soc.*, 50, 3318 (1928).
59. von Mutzenbecher, E.: *Biochem. Z.*, 266, 226, 250, 259 (1933).
60. Adair, R., and Robinson, S.: *Biochem. J.*, 24, 1864 (1930).
61. Block, R. J.: *J. Biol. Chem.*, 103, 455 (1934).
62. Chick, H.: *Biochem. J.*, 8, 404 (1914).
63. Moore, N. S., and van Slyke, D. D.: *J. Clin. Investig.*, 8, 337 (1929).
64. Kerr, W. I., Hurwitz, S. H., and Whipple, G. H.: *Am. J. Physiol.*, 47, 356, 370, 379 (1918).
65. Whipple, G. H., et al.: *Am. J. Physiol.*, 52-54 (1920).
66. Whipple, G. H.: *Physiol. Rev.*, 2, 460 (1922).
67. Leiter, L.: *Medicine*, 10, 135 (1923).
68. Leiter, L.: *Arch. Internal Med.*, 48, 1, 286 (1931).
69. Maver, M. B.: *J. Am. Assoc.*, 74, 934 (1932).

Blood Coagulation

70. Pickering, J. W.: *The Blood Plasma in Health and Disease* (1928).
71. Pickering, J. W.: *Brit. J. Exper. Biol.*, 2, 397 (1925).
72. Wöhlisch, E.: *Ergebnisse Physiol.*, 28, 443 (1929).
73. Moore, N. S.: In Oppenheimer's *Handbuch der Biochemie*, 4, 44 (1925).
74. Morawitz, P.: *Ergebnisse Physiol.*, 4, 307 (1904).
75. Fuchs, H. J.: In Nord and Weidenhagen's *Ergebnisse d. Enzym Forschung*, 2 (1933).
76. Mills, C. A.: *Am. J. Med. Sci.*, 172, 501 (1926).
77. Mills, C. A.: *Chinese J. Physiol.*, 1 (1927).
78. Howell, W. H.: (Pasteur Lecture) *Proc. Instit. Med. Chicago*, 5, 139 (1924-25).
79. Howell, W. H.: *Harvey Lectures*, 12, 273 (1917).
80. Bordet, J.: *Ann. d. l'Inst. Pasteur*, 34, 561 (1920).
81. Nolf, P.: In Gilbert and Weinberg's *Traité du Sang* (1913).
82. Schmidt, A.: *Zur Blutlehre* (1893).
83. Schmidt, A.: *Weitere Beiträge zur Blutlehre* (1895).
84. Wooldridge, L. C.: Collected papers on *The Chemistry of the Blood* (1893).

CHAPTER XVI

THE CARRIAGE OF THE BLOOD GASES AND THE ACID-BASE EQUILIBRIUM OF THE BLOOD

THE blood transports oxygen to the tissues and then returns to the lungs for an additional supply of oxygen. At the same time the blood loses in the lungs the carbon dioxide gained in the tissues. The functions involved in this exchange of oxygen and carbon dioxide must be attributed to the substances which constitute the blood. Therefore an examination of the elements which form the blood is the first step in the understanding of the carriage of the blood gases. The blood is composed of formed elements, chiefly red blood corpuscles, and of fluid plasma in which the cells are suspended. Both cells and plasma are formed largely of water, 72 Gm. per cent and 93.5 Gm. per cent respectively. The cells contain hemoglobin (33 Gm. per cent), a compound of the protein, globin, and a heme consisting of four pyrrol nuclei and iron.*

| Constituents of blood. | Milliequivalents per 100 cc. | |
|-------------------------------|------------------------------|---------|
| | Cells. | Plasma. |
| Na..... | ≡ | 135 |
| K..... | 108 | 5 |
| HCO ₃ | 18.2 | 27.2 |
| Cl..... | 54 | 101 |
| Mg..... | 3 | 1 |
| Ca..... | 0 | 3 |
| SO ₄ | ≡ | 1 |
| PO ₄ | 2 | 2 |
| Organic PO ₄ | 17 | trace |

(Unpublished data from the laboratory of J. P. Peters.)

In human beings the chief cation of the red blood cell is potassium. In addition there is a small amount of magnesium. Among the anions are bicarbonate, chloride, sulphate, and phosphate. The plasma contains only a small amount of albumin, globulin and fibrinogen which together form approximately 7 Gm. per cent. The inorganic anions

* 100 cc. of blood contain, in addition, 4 Gm. of other solids, so that the total weight is 109 Gm. Similarly, 100 cc. of plasma contain 2 Gm. of solids other than proteins, making a total weight of 102 Gm.

are the same as those of the corpuscles, for the envelop of the red blood cells is permeable to these anions. However, there is a segregation of the cations, the surface of the cells not being permeable to them. Sodium, for practical purposes, may be considered as the only one that appears in the plasma, though small amounts of calcium, magnesium, and even potassium are also present. In what manner do these substances of the blood interact to facilitate the gaseous exchanges which take place in the pulmonary and tissue capillaries?

Oxygen.—Obviously, the oxygen may exist in the blood in two forms: In physical solution and in chemical combination. The volume of a gas dissolved in any fluid depends upon the partial pressure of the gas. Oxygen forms about one fifth of the volume of the gases of the air; and since the total pressure of the atmosphere is 760 mm. Hg, at sea level, the partial pressure of oxygen is approximately 150 mm. Hg. In an atmosphere of pure oxygen, blood dissolves 2.2 volumes per cent. Hence with blood in contact with air, 0.4 volume per cent are present in physical solution. However, the partial pressure of oxygen in the arterial blood is 80 mm. Hg. Therefore, only 0.2 volume per cent of oxygen is carried in physical solution in arterial blood. Approximately one hundred times as much may be held in chemical combination as oxyhemoglobin. The manner by which the oxygen carrier, hemoglobin, affords tissues oxygen in amounts adequate to support life must therefore be examined.

Whereas the volume of oxygen in physical solution varies with the partial pressure of the gas, this relationship holds true for the combination of oxygen with hemoglobin only between the pressure of 0 and 150 mm. Hg. At the partial pressure of oxygen in the air, hemoglobin holds the maximum volume of which it is capable, approximately 20 volumes per cent. On increasing the partial pressure of oxygen, no further formation of oxyhemoglobin can take place. The hemoglobin is therefore 100 per cent saturated at 150 mm. Hg pressure of oxygen. This is called the *oxygen capacity* of the blood. Though an increase of partial pressure of oxygen above 150 mm. Hg has no effect, a diminution below 150 mm. Hg causes a breakdown of the labile compound, oxyhemoglobin. For example, at 80 mm. Hg partial pressure of oxygen or that of the arterial blood, hemoglobin holds 19 volumes per cent and is therefore 95 per cent saturated. While at 35 mm. Hg, the partial pressure of oxygen in mixed venous blood, it holds 14 volumes per cent of oxygen and is therefore 70 per cent saturated.

By determining the volume of oxygen held in combination with hemoglobin at various partial pressures of the gas and plotting the tensions as abscissae and the percentages of saturation as the ordinates a smooth "S"-shaped oxygen dissociation curve may be drawn. This curve which indicates the volume of oxygen held by hemoglobin at all partial pressures occurring in the body, shows that as the partial pressure diminishes by 20 mm. Hg, a greater volume of oxygen (8 volumes per cent) is liberated between 40 and 20 mm. Hg than between 90 and

70 mm. Hg (0.5 volume per cent); that is, more oxygen is liberated in the tissues with lower oxygen tension and therefore greater oxygen requirement. The oxygen dissociation curve is sensitive to changes of pH . Alkali increases the affinity of hemoglobin for oxygen. This may be due in part to the fact that oxyhemoglobin is more acid than hemoglobin. Hence, by increasing the alkalinity of the blood more oxyhemoglobin is formed. Acid, on the other hand, decreases the affinity of hemoglobin for oxygen. For a given tension of oxygen, the concentration of oxyhemoglobin diminishes as the blood becomes less alkaline. Thus during exercise the arterial blood at a partial pressure of 30 mm. Hg may be 45 per cent saturated, instead of 60 per cent, and therefore yield an additional 3 volumes per cent for the use of the tissues (Fig. 21).

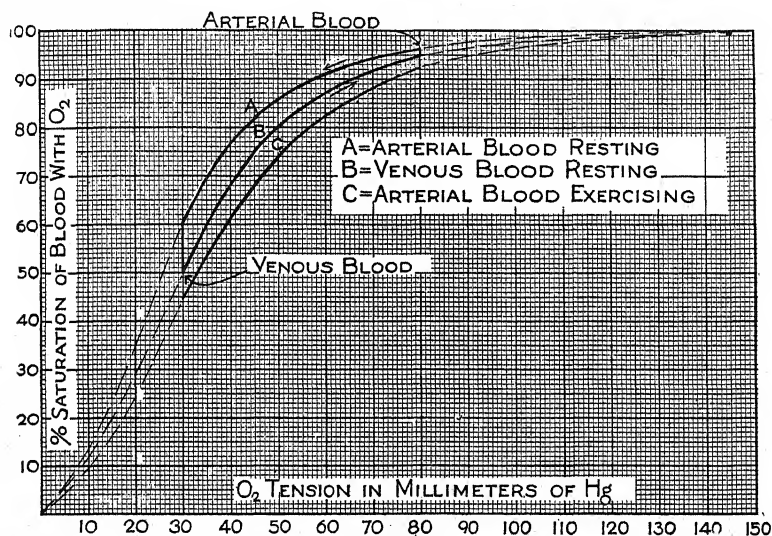


Fig. 21.—Oxyhemoglobin dissociation curves indicating changes occurring in venous blood during rest and arterial blood in exercise.

Carbon dioxide has two synergistic effects on oxyhemoglobin: It not only diminishes the volume of oxygen combined with hemoglobin by virtue of its acid properties but also as a result of the formation of a compound termed *carbhemoglobin*¹ by Henriques. Carbhemoglobin, like acid, diminishes the ability of hemoglobin to retain oxygen. Therefore, as carbon dioxide diffuses into the capillary blood, the saturation of hemoglobin may diminish from 60 to 50 per cent; an additional 2 volumes per cent of oxygen are liberated (Fig. 21). Thus, in the acquisition or liberation of oxygen in chemical combination three mechanisms are involved: One is observed in the effects of changes of partial pressure; a second, in those produced by changes of the pH of the blood; and the third is the reversible formation of carbhemoglobin.²*

* For a discussion of the factors influencing the diffusion of oxygen see Himwich and Barr.³

Carbon Dioxide.—Arterial blood contains about 50 volumes per cent of carbon dioxide, and the blood receives an additional amount as it passes through the tissue capillaries. Like oxygen, only a small volume of carbon dioxide is dissolved in physical solution, by far the greater part is in chemical combination. The volume dissolved depends upon the partial pressure of carbon dioxide and may be plotted in a straight line (Fig. 22). In an atmosphere of pure carbon dioxide, blood dissolves 51.1 volumes per cent of that gas. Hence at 40 mm. Hg tension, or that of the carbon dioxide of the arterial blood, the volume in physical solution is $51.1 \times 40/760 = 2.7$ volumes per cent. Therefore, approximately twenty times as much carbon dioxide is retained in chemical combination. By determining the volume of carbon dioxide held at different tensions and plotting these values, a smooth curve like that of the figure may be obtained. The distance between the

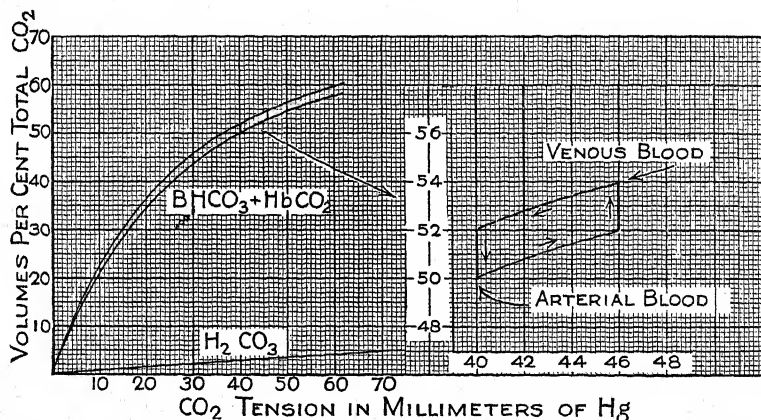


Fig. 22.—At left, carbon dioxide dissociation curves of arterial and venous blood; at right, changes occurring during respiration (presented in larger units).

straight line and the curve represents the carbon dioxide in chemical combination; and the total volume of carbon dioxide held at 40 mm. Hg tension may be termed either the carbon dioxide capacity or the alkaline reserve of the blood⁴ (Fig. 22).

The carbon dioxide dissociation curve resembles that of oxyhemoglobin in so far as it is raised by alkali and lowered by acid. The addition of base renders it available for combination with carbon dioxide. On the other hand, acids competing with carbon dioxide unite with some of the base and leave less for combination with carbon dioxide, thus decreasing the alkaline reserve. Since oxyhemoglobin is a stronger acid than hemoglobin, the formation of oxyhemoglobin in the pulmonary capillaries attracts base from bicarbonate, liberating additional carbon dioxide. This effect of the entrance of oxygen into the blood (discovered by Christiansen, Douglas and Haldane⁵), was thought to be due entirely to the greater acidity of oxyhemoglobin. However,

it is possible that part of the effect of oxygen is exerted on the compound carbhemo-globin. In 1928 Henriques¹ pointed out that only a small fraction of the carbon dioxide could be eliminated during the short interval required for the passage of the blood through the pulmonary capillaries if all the carbon dioxide released in respiration came from bicarbonate, the time for the formation of anhydrous carbon dioxide being the limiting factor. Henriques found that from a solution of hemoglobin *in vacuo*, carbon dioxide was first given off quite rapidly and then more slowly. The rapid release of carbon dioxide he attributed to the splitting of a compound with hemoglobin which he called carbhemo-globin. Part of the effect of the oxygenation of the blood may therefore be due to the influence of oxyhemoglobin on carbhemo-globin.

Later work of Meldrum and Roughton⁶ and Stadie and O'Brien⁷ revealed that the slow release of carbon dioxide in Henriques' experiments must have been the result of the inactivation of an enzyme, carbonic anhydrase, which accelerates the dehydration of carbonic acid. The large volume of carbon dioxide that leaves the blood in the pulmonary capillaries is made possible by the action of this enzyme. The reverse processes occur as blood becomes venous in character.

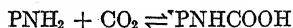
CALCULATION OF RELATIVE CONCENTRATION OF HbCO_2 IN HUMAN BLOOD AT REST
FROM DATA OF HENDERSON*²

| | Arterial. | | Venous. | | Δ = venous-arterial. | | |
|--|-----------|--------|---------|--------|-----------------------------|--------|--------------|
| | Serum. | Cells. | Serum. | Cells. | Serum. | Cells. | Whole blood. |
| pH. | 7.421 | 7.205 | 7.399 | 7.157 | | | |
| CO_2 combined, mm. per liter. | 15.29 | 6.03 | 16.07 | 6.65 | 0.78 | 0.62 | 1.4 |
| HCO_3 , mm. per liter. | 15.29 | 4.59 | 16.07 | 4.68 | 0.78 | 0.09 | 0.87 |
| HbCO_2 , mm. per liter. | | 1.44 | | 1.97 | | 0.47 | 0.47 |
| $\text{HbCO}_2/\text{CO}_2$ combined. | | 0.25 | | 0.30 | | 0.76 | 0.33 |

* Henderson, L. J., *Blood, a Study in General Physiology* (1928), p. 201.

The tension of carbon dioxide may increase from 40 to 46 mm. Hg as the blood passes through the tissue capillaries (Fig. 22). Five per cent of the carbon dioxide that has entered the blood is retained in physical solution. In what manner is the remaining carbon dioxide held in chemical combination? The carbon dioxide, like the oxygen, is held in a labile combination sensitive to changes of the partial pressure of that gas. However, in the case of carbon dioxide, the labile combination is due, only in part, to the formation of a compound with hemoglobin, but chiefly to the fact that carbon dioxide is an acid and therefore competes with other acids for the cation available in the blood.

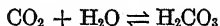
Thus carbon dioxide is held both as carbohemoglobin and bicarbonate. Although the total volume of carbon dioxide carried as carbohemoglobin is not large, being approximately 5 to 10 per cent of that carried as bicarbonate, nevertheless one third of the difference of the carbon dioxide contents of arterial and venous blood is due to the formation and breakdown of carbohemoglobin, the latter compound undergoing cleavage in the presence of oxygen. As oxygen leaves the blood, greater amounts of carbohemoglobin are formed. The combination of carbon dioxide with hemoglobin to form carbohemoglobin may be in the nature of a carbamate.



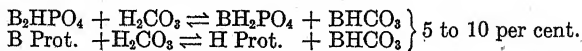
The carbon dioxide carried as bicarbonate obtains its base in several ways. As the partial pressure of carbon dioxide increases in the tissue capillaries, it reacts with alkaline phosphate to form basic phosphate and bicarbonate. During the rise in tension, carbon dioxide also robs the serum proteins of some of their base. Both of these reactions, however, account for no more than 5 to 10 per cent of the carbon dioxide absorbed in chemical combination. Approximately 60 per cent of carbon dioxide is carried because of the base released by hemoglobin. This base is supplied as a result of two different reactions. Probably 10 to 15 volumes per cent of the carbon dioxide are absorbed as bicarbonate by direct competition with oxyhemoglobin and hemoglobin for the base held in chemical combination with these proteins. Oxyhemoglobin is more acid than hemoglobin, and approximately half of the carbon dioxide received by the blood in the tissue capillaries combines with the base released in the change of oxyhemoglobin to hemoglobin. Thus hemoglobin not only carries the oxygen of the blood but is largely responsible for the transport of carbon dioxide.

Carbon dioxide entering the blood in the tissue capillaries is partitioned as follows:

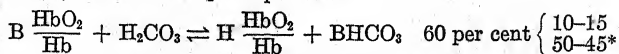
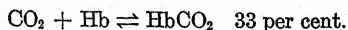
(a) Physical solution:



(b) Chemical combination:



Hemoglobin directly or indirectly carries 90 to 95 per cent of carbon dioxide:



* 10-15 per cent by direct competition of CO_2 , Hb, and HbO_2 for base; 50-45 per cent as result of the reaction $\text{BHbO}_2 \rightleftharpoons \text{HHb}$.

The exact amount of HbCO_2 in the blood is not known. According to Ferguson and Roughton (*J. Physiol.*, 83, 87 (1934)) only 15 per cent of the CO_2 transported occurs in the form of HbCO_2 . If this value is correct, then 75-80 per cent of CO_2 is carried by base released by hemoglobin; approximately 15 per cent of the latter by direct competition and 60-65 per cent by virtue of the base liberated as Hb releases O_2 in the tissues.

for combination with carbon dioxide than has the plasma, for more base is held in combination with the greater amount of protein in the cell. As a result, at first more potassium bicarbonate is formed in the cell than is sodium bicarbonate in the plasma. Thus there is an excess of the bicarbonate ion in the cell, and this substance therefore diffuses out of the cell into the plasma. This process cannot continue unless some other anion diffuses from the plasma to the corpuscle.

The chief anion which is displaced in the plasma is chloride, so that the chloride ion that was formerly combined with sodium in the plasma now forms potassium chloride in the cell. This exchange constitutes the *chloride shift*. As a result of this shift, additional sodium bicarbonate is formed and the final ratio of the bicarbonate of the cell to that of the plasma is approximately as 2 is to 3. Perhaps half the carbon dioxide carried chemically obtains its base because of this exchange of anions.

CHLORIDE SHIFT CAUSED BY ENTRANCE OF CARBON DIOXIDE IN BLOOD

| Corpuscles. | Plasma. | Tissues. |
|---|--|---------------|
| 1. $\text{KHbO}_2 + \text{H}_2\text{CO}_3 = \text{KHCO}_3 + \text{HHb} + \text{O}_2$ | $\text{Na proteinate} + \text{H}_2\text{CO}_3 = \text{NaHCO}_3 + \text{H prot.}$ | CO_2 |
| 2. $\text{K} \xrightarrow{\quad} \text{HCO}_3^-$ $\text{Cl}^- \xleftarrow{\quad} \text{Na}^+$ $\text{H}_2\text{O} \xleftarrow{\quad}$ | | |

The potassium oxyhemoglobinate of the cell is but poorly ionized. The potassium in inorganic combination as potassium chloride or potassium bicarbonate, on the contrary, is well ionized and consequently exerts an osmotic pressure much greater than that of the organic form. Therefore, as the blood passes through the tissue capillaries, the cells swell since water diffuses into them from the plasma. On the other hand, the cells shrink as the blood becomes arterialized and potassium oxyhemoglobinate is formed again.

It may be of some interest to present a brief summary of a paper by van Slyke, Wu and McLean,⁸ in which the equilibria involved are treated in a manner different from that just described. Equilibrium requires conformation to three basic laws: (a) In cells and serum positive and negative ions must balance; (b) the ratio $\frac{(\text{HCO}_3)_c}{(\text{Cl})_c} = \frac{(\text{HCO}_3)_s}{(\text{Cl})_s}$; (c) the total osmotic pressures exerted by the constituents of cells and serum must be equal. In A (Fig. 23) is seen the blood at pH of 7.8 with more base and protein (hemoglobin) in the cell and more chloride and bicarbonate in the serum. Then the carbon dioxide tension is increased, for purposes of illustration, until the pH falls below physiologic values to 6.6, the iso-electric point of oxyhemoglobin, and the base formerly bound by hemoglobin has shifted to bicarbonate and

chloride. In B only the first of the three conditions is fulfilled. Positive and negative charges balance; but the greatly increased concentration of bicarbonate in the cells violates the second law; similarly, the osmolar concentration of the cells exceeds that of the serum. In order to restore electrolyte distribution in conformity with Donnan's law, the bicarbonate ion migrates from cells to serum and the chloride ion in the reverse direction (C). Finally, in D osmotic equilibrium is also restored as water migrates from serum to cells. The processes occur simultaneously even though they are represented as occurring in successive steps.

This extreme and unphysiologic example is presented because it shows clearly the conformity of the equilibria of the blood to the three

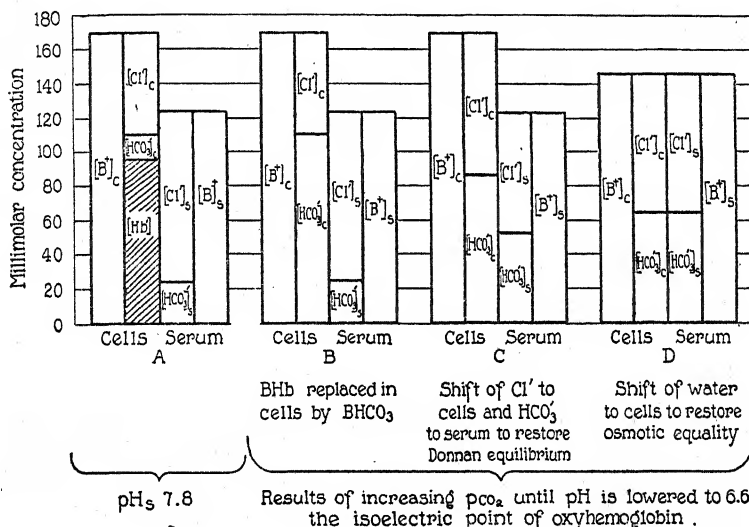


Fig. 23.—Van Slyke, Wu, and McLean in *J. Biol. Chem.*, 56, 1923. Effects of carbon dioxide tension changes on the electrolyte and water distribution of oxygenated blood.

fundamental laws. It should be pointed out that these diagrams were drawn before the discovery of carbohemoglobin, which is therefore not included. Actually, it is well known that $\frac{(\text{CO}_2 \text{ combined})_c}{(\text{Cl})_c} = 1.23 \frac{(\text{CO}_2 \text{ combined})_s}{(\text{Cl})_s}$; i. e., there is more combined carbon dioxide in the cell than can be accounted for by the Donnan equilibrium. Perhaps the excess carbon dioxide can be explained by the portion bound as carbohemoglobin. Furthermore the ratio of the hydrogen ions in serum and cells is smaller than that of the chloride ions in cells and serum. Whatever the explanation may be for these deviations from the Donnan ratios, experiments of van Slyke, Hastings, Murray and Sendroy⁹ revealed the following relations:

$$\frac{(\text{A.H.})_s}{(\text{A.H.})_c} = 0.77 \frac{(\text{Cl})_c}{(\text{Cl})_s} = 0.62 \frac{(\text{CO}_2 \text{ combined})_c}{(\text{CO}_2 \text{ combined})_s}$$

The foregoing discussion discloses that a reciprocal action exists between oxygen and carbon dioxide, the entrance of one into the blood assisting the exit of the other and *vice versa*. These effects of oxygen and carbon dioxide upon each other are due to the reciprocally antagonistic actions of oxyhemoglobin and carbohemoglobin, and also to the competition of the acids (carbon dioxide and oxyhemoglobin) for base. The oxygen entering the pulmonary capillaries forms oxyhemoglobin. This, in turn, attracts base from bicarbonate and releases carbon dioxide bound chemically both as carbohemoglobin and as bicarbonate, the carbon dioxide arising from bicarbonate leaving the blood with

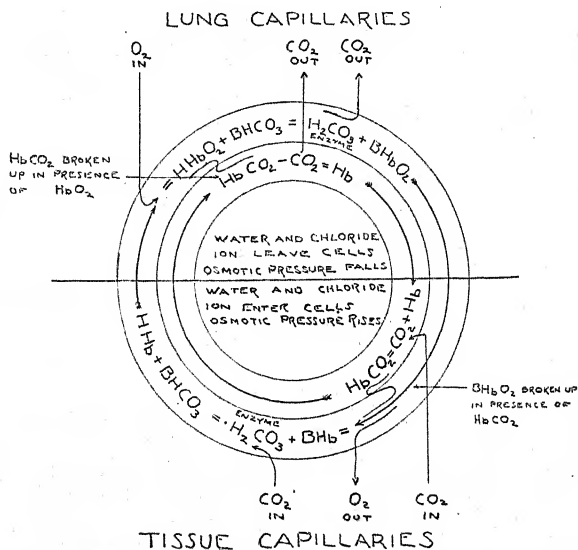


Fig. 24.—Exchanges occurring during the respiratory cycle.

the aid of the enzyme, carbonic anhydrase. The carbon dioxide that leaves the blood, moreover, yields base for the formation of additional basic oxyhemoglobin. With the loss of carbon dioxide from the blood, chloride and water leave the cell and the osmotic pressure falls.

In the tissues oxygen leaves oxyhemoglobin, thus facilitating the formation of carbohemoglobin, and, in addition, liberating base from the weaker acid, hemoglobin—base which then unites with carbonic acid to form bicarbonate. At the same time, the entrance of carbon dioxide into the blood attracts base from oxyhemoglobin, thus expediting the release of additional oxygen. As carbon dioxide enters the blood, chloride and water shift to the cell and its osmotic pressure rises.

Caisson Disease.—The chemical adaptations of the blood facilitate the exchanges of the large volumes of oxygen and carbon dioxide neces-

sary for the maintenance of mammalian life. It is instructive to contrast the exchange of these gases with those of nitrogen, which is metabolically inert and is retained in physical solution only. The volume of nitrogen physically dissolved like that of oxygen or carbon dioxide is increased in proportion to the atmospheric pressure. When a diver is gradually lowered in a diving bell, the pressure within it must be increased to that of the column of water above it. Accordingly, air is pumped into the bell until the pressure of the gases is sufficient to counteract that of the column of water. The diver becomes readily adapted to the high pressure after some passing discomfort, such as a feeling of fullness in the head, ringing in the ears and dizziness. However, the return to the surface is hazardous. The excess of physically dissolved oxygen and carbon dioxide which is liberated as the pressure is diminished produces no ill effects. The oxygen is taken up by the hemoglobin until it is used for the metabolism of the tissues. The carbon dioxide forms bicarbonate and is eliminated in the lungs in the usual manner.

However, there is no mechanism for the rapid excretion of nitrogen, since it enters into no chemical combination with the substances of the blood. If the decompression is not performed slowly, bubbles of nitrogen are formed in the lipoidal tissues, just as charged seltzer water forms bubbles as the pressure within the bottle is diminished. The particular site of the bubbles is due to the fact that nitrogen is more soluble in lipoidal tissues than in the blood, which therefore is unable to absorb sufficient nitrogen to prevent the formation of bubbles in the fatty portions of the body. When nitrogen bubbles are formed in the central nervous system and the joints, the syndrome of caisson disease or the "bends" develops. This consists of paralysis and pains varying in locations according to those of the bubbles.

Acidosis and Alkalosis.—Though normal quiet breathing during rest is carried on as a result of a complex interchange, nevertheless the change of pH is minimal. This minimal variation from 7.40 to 7.38 which occurs as the blood passes through the tissue capillaries is made possible by two mechanisms: The buffer action of the blood and the change from oxyhemoglobinate to hemoglobinate. A *buffer action* is one in which a strongly dissociated acid is replaced by a weaker one. In this process, the salt of the acid is formed and, most important, the increase in hydrogen ion is greatly diminished. For example, the comparatively highly dissociated carbonic acid reacting with basic oxyhemoglobinate forms bicarbonate and the more weakly dissociated acid hemoglobinate. In addition the formation of the salt, bicarbonate, depresses the ionization of the remaining carbonic acid. Serum proteinate and dibasic phosphate also participate in these processes. The entrance of carbon dioxide into the blood tending to make it more acid is counteracted by the exit of oxygen; for reduced hemoglobinate is less acid than is oxyhemoglobinate.

The *hydrogen ion concentration of the blood* may be determined

directly by electrometric methods¹⁰ and also indirectly. When an acid dissolves in water, it dissociates to a certain extent into anions and cations, $(HA) = (H^+)(A^-)$. With an acid at a given concentration, a definite and constant amount of dissociation occurs; $K = \frac{(H^+)(A^-)}{HA}$.

This may be written $H^+ = K \frac{HA}{A^-}$. In the case of the weak acid, carbonic acid, the dissociated A^- comes from the bicarbonate salt. If the degree of dissociation of the salt be represented by λ and $\frac{K}{\lambda}$ be considered constant, then K/λ may be designated as K_1 and $H^+ = K_1 \frac{H_2CO_3}{BHCO_3}$; or, logarithmically, $\log H = \log K_1 + \log \frac{H_2CO_3}{BHCO_3}$, the *Hasselbalch-Henderson equation*. Expressed in negative logarithms, $-\log H = -\log K_1 - \log \frac{H_2CO_3}{BHCO_3}$; or, in Sørensen's notation, $pH = pK_1 + \log \frac{BHCO_3}{H_2CO_3}$.

The total volume of carbon dioxide of the blood is determined by direct analysis, and is represented in the numerator of the last term of the equation. Carbonic acid may be calculated for any given tension of carbon dioxide. The value of the last term of the equation is thus obtained and is usually 1.28. This with a pK_1 of 6.12 yields a pH of 7.40. A change either in the numerator or denominator of the last term will produce a corresponding alteration of pH . These changes may be considered nonlogarithmically; i. e., $H^+ = K \frac{H_2CO_3}{BHCO_3}$.

It is obvious that the concentration of the hydrogen ion is raised either when carbonic acid increases (for example, in rebreathing carbon dioxide) or bicarbonate decreases, because of the accumulation of a fixed acid in the blood; i. e., lactic acid during exercise. Similarly, the loss of fixed base in the urine during nephritis or in the stool during diarrhea may produce an acidosis. On the other hand, if carbon dioxide is pumped out of the body by overbreathing or bicarbonate of the blood is increased by the ingestion of bicarbonate, the hydrogen concentration will decrease. Prolonged vomiting results in the loss of chloride from the body. This permits bicarbonate to increase at the expense of chloride; and despite some loss of base, an alkalosis supervenes. These phenomena may be represented graphically.

In the figure the point N on the carbon dioxide dissociation curve is in a position characteristic of the normal pH . If an increased amount of carbon dioxide is inspired, the pH decreases to 7.3 at A. Similarly, as an acid is liberated in the blood stream which robs bicarbonate of its base N assumes the position of D. Again, if overbreathing occurs, the carbon dioxide tension will be decreased and N shifts to B with a pH of 7.5. The ingestion of bicarbonate produces a rise in the carbon dioxide dissociation curve, N moves to C and the pH becomes 7.5.

The changes just described never occur without compensatory reac-

tions since the body acts in such a way that it maintains the constancy of the environment of its cells. Perhaps the highly specialized cells of the mammalian body require an optimum medium for their best function. The response of the body to an increased concentration of carbon dioxide in the inspired air is overbreathing. By this hyperpnea the carbon dioxide is pumped out and the pH raised to a normal level. On the other hand, if overbreathing is induced voluntarily and carbon dioxide is thus removed from the blood, a period of apnea finally develops during which carbon dioxide again accumulates and the pH falls to a more normal level.

Respiratory Response to Change of pH .—The most frequent cause of acidosis in the healthy individual is physical exertion, since during exercise lactic acid accumulates in muscle and is poured into the blood.

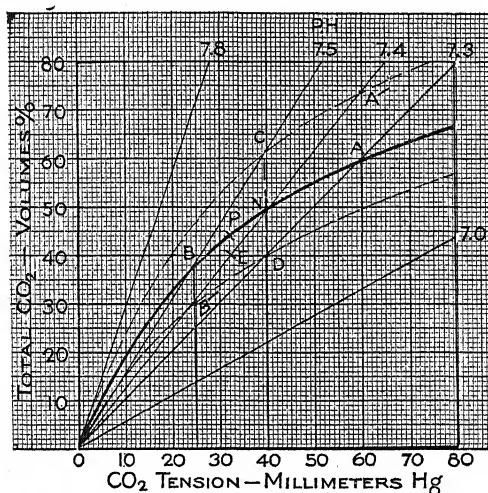


Fig. 25.—Effect of changes of H_2CO_3 and $BHCO_3$ on the pH of blood.

The bicarbonate of the blood is depleted as sodium lactate is formed and preformed carbon dioxide is liberated. This produces an acidosis despite the hyperpnea which causes the elimination of increased amounts of carbon dioxide by the lungs, since the decrease in the numerator of the fraction $\frac{H_2CO_3}{BHCO_3}$ is not sufficient to compensate for the fall of the alkaline reserve of the blood.

The decrease in pH occurring during exercise is in accordance with the conception that the respiratory centers react to the carbon dioxide and pH of the arterial blood. The quality of the blood is an important influence in determining the action of the respiratory centers. However, a closer study reveals that the respiratory centers are sensitive to the concentration of carbon dioxide and hydrogen ion within the centers, the quality of the arterial blood being only one of the factors

determining the concentration of these substances. The effects of the rate of blood flow through the respiratory centers and the carbon dioxide produced by their own metabolism are also of importance.

Within certain limits, respiration is depressed by an increased cerebral blood flow; for the carbon dioxide produced by the metabolism of the centers is thus removed more effectively. The great importance of the intrinsic carbon dioxide production is seen in the respiratory depression that may follow a marked increase in cerebral blood flow even during carbon dioxide inhalation. On the other hand, a decreased cerebral flow, with less efficient removal of the carbon dioxide produced by the centers, is followed by an augmented respiration.¹¹

Such a conception can explain the results obtained by analyses of arterial blood drawn during exercise.¹² The pH of the blood before exercise was 7.35, and the pulmonary ventilation 5 liters per minute. During the last minute of three and one-half minutes of peddling on a stationary bicycle, the pH of the arterial blood going to the centers fell to 7.28, and the respiratory volume increased to 90 liters per minute. However, during the first three minutes of rest the pH fell to 7.15 as the lactic acid which had accumulated in the muscles continued to enter the blood. Nevertheless, the respiratory volume did not continue to increase; on the contrary, it fell to 30 liters per minute. Perhaps the greater acidity of the blood produced a vasodilatation of the vessels of the brain; so that the flow of blood through the respiratory centers was increased. If such be the case, then the carbon dioxide produced by the metabolism of the cells constituting the respiratory centers would be carried away more readily, thus reducing the tension of carbon dioxide and therefore the activity of the centers.¹³ Similarly, the augmented respiration following the injection of alkali may be imputed to cerebral vasoconstriction. Certainly the activity of the respiratory centers is not governed by the quality of the arterial blood alone. During nephritis, respiration is not increased despite an existing acidosis.

Renal Adjustment to Change of pH.—The kidney, like the respiratory centers, is essential in maintaining the concentration of hydrogen ion of the body. The actions of the kidney in particular instances of disturbed acid-base equilibria will be detailed below. However, the mechanisms underlying this renal function will now be described. Usually more acids (phosphate, chloride, sulfate, organic) than bases (potassium, sodium, calcium, ammonia) are excreted by the kidney, the average pH of urine being 6. This is of significance, for both the inorganic and the organic acids may accumulate in the body during the various stages of the intermediary metabolism of the three food-stuffs. Three mechanisms function to yield an acid urine. First may be mentioned the change of dibasic to monobasic phosphate. In this manner, one of the two molecules of base of the phosphate compound is saved by the kidney. At the normal pH of the blood, 7.4, 20 per cent of inorganic phosphate exists as monobasic phosphate; while in

the urine with pH 6, 85 per cent is in the form of the acid salt; and during an acidosis, a urine with pH 5 contains 98 per cent of phosphate as the acid salt.¹⁴

A second mechanism is concerned with the excretion of organic acids as such and not as salts of fixed base, thus avoiding a loss of base. In a urine with a pH less than 6, 10 to 20 per cent of organic acids may be excreted in this form. A third important method for the conservation of base during acidosis occurs in the elaboration of ammonia by the kidney, and the substitution of ammonia for fixed base in the formation of the salts excreted in the urine.¹⁵ This last mechanism is sensitive not only to an excess of fixed acid, but also fixed base; for ammonia formation is suppressed during alkalosis. Though the elaboration of ammonia is also reduced during the carbon dioxide deficit of hyperventilation, its formation is not significantly increased by the acidosis caused by the retention of carbon dioxide. Normally some ammonia salts are always excreted so that during chronic interstitial nephritis, when the kidney may lose the ability to form ammonia, an acidosis develops because of the excretion of salts of fixed base.

A specific example of the reaction of the kidney to an excess of acid may be examined in the renal response to the lactic acidosis of exercise. In the first place, whenever the renal threshold of 30–40 mg. per cent is exceeded, lactate ion is excreted in the urine¹⁶ as a salt of fixed base; second, and more important, part of the lactic acid is excreted as ammonia salt;¹⁷ and third, lactic acid is also eliminated in the acid form without any base.

During alkalosis additional fixed base is excreted, partly because of its greater concentration in the blood, and in part because of the reversal of the three renal mechanisms just described. With an alkaline urine, dibasic phosphate is excreted at the expense of monobasic phosphate, organic acids are eliminated entirely as salts and not in the form of free acids and ammonia formation is inhibited. Excess base is also excreted as bicarbonate, while during acidosis only negligible amounts of bicarbonate leave through the kidney.

Since the normal kidney responds effectively either to an acidosis or to an alkalosis, the method of choice in the treatment of either condition is the injection or ingestion of saline solutions. In the case of acidosis, sodium will be retained and, provided the water intake of the body is sufficient, chloride will be eliminated. On the other hand, during alkalosis sodium will be excreted in the urine and chloride will be retained. In this manner the kidney will tend to restore the normal pattern of the blood.

Responses of Various Viscera to Changes of pH.—Many organs are involved in the maintenance of the hydrogen ion concentration of the blood. The liver plays an important rôle in this respect, for with the formation of urea from the ammonia produced in the deamination of the amino acids, the action of a comparatively strong base is eliminated without neutralization by an acid.

The liver is also effective in reducing the lactic acid of the blood. It has been demonstrated that during rest and exercise the liver continues to absorb lactic acid from the blood.¹⁸ However, not only the liver but the heart also,^{19, 20} the brain,^{21, 22} and resting muscle²³ all remove lactic acid from the blood. The skin also functions to relieve a lactacidosis.²⁴ Fixed base is saved in this process, because unionized lactic acid appears in the perspiration.²⁵

In general, by the removal of lactic acid the organs act to maintain the pH of the blood. However, the effects of the removal of lactic acid on the acid-base equilibria vary for the different organs. The oxidation of lactic acid by the brain, for example, may diminish instead of increase the alkalinity of the blood, since it results in the production of three molecules of carbon dioxide for each molecule of lactic acid removed. Nevertheless, an advantage does accrue in this process, since for lactic acid is substituted a volatile acid which is eliminated by the lungs without loss of base. In the excretion of lactic acid by the skin and kidney, an increase of pH is gained at the cost of the loss of a calorific substance. The liver, muscle and perhaps also the heart convert lactic acid to a neutral substance, carbohydrate, and at the same time cause the liberation of the base formerly bound as the lactate salt. The continuous effect of the liver on the pH of the blood should be approximately an increase of 0.03.²⁶ Since insufficient oxidations in muscle result frequently in an accumulation of lactic acid in the blood, the removal of that substance by other parts of the body provides a corrective mechanism against the acidosis arising from impaired oxidations.

Accumulation of Organic Acids During Alkalosis.—During alkalosis lactic acid accumulates in the blood,²⁷ and thus tends to counteract the diminution of the hydrogen ion concentration.^{28, 29} The lactic acid of the blood is produced during rest by the blood itself as a result of glycolysis, the splitting of glucose to lactic acid. The level of blood lactic acid during rest is therefore the resultant of the new formation of that substance in the blood and its removal by the various organs. Any process which tends to increase the formation or diminish the removal of lactic acid would act in a protective manner during an alkalosis. Alkalosis accelerates the formation of lactic acid in all tissues. However, unless the alkalosis is extreme, the various organs are probably not only capable of reconvertng their own extra lactic acid to carbohydrate, but of continuing to remove it from the blood. It is therefore significant that the rate of glycolysis of the blood increases with its pH , as the blood has no effective mechanism for the oxidative removal of that substance.^{30, 31} In this manner the alkalosis of itself causes the changes which tend to counteract the diminished hydrogen ion concentration.

The acid ketone substances also accumulate in the blood during alkalosis.³² Perhaps the increase of pH changes the ratio of food-stuffs oxidized, so that more fat and less carbohydrate are burned.

Whatever the exact mechanism of the ketogenesis during alkalosis may be, it represents another example of adherence of the body to an established pattern.

Integrative Action of the Body.—The responses of the body to changes of the acid-base equilibrium have been treated in a relatively simple fashion, isolated from the other bodily reactions. However, in the maintenance of the internal environment, the fluid matrix of the body, many equilibria in addition to that of pH are involved; for example, the concentrations of oxygen, of the various ions and of water are maintained optimal for the cells. When one equilibrium is changed, all the others may be secondarily affected to a greater or lesser degree; so that the response of the body becomes a manifold one, an integration of the changes of many equilibria. Examples of such integrated responses will now be described; and the first to be considered are those occurring during emphysema.

Emphysema.—Despite the hyperpnea, the patient may have an inadequate exchange of gases in the lungs; so that the arterial blood is poorly oxygenated. The greater amount of reduced hemoglobin will give it a bluish color, cyanosis, which may be detected in the lips, cheeks, ears, and tongue. The carbon dioxide content of the blood may also be increased. This tends to raise the tension of carbon dioxide in the body and to diminish the pH. N is displaced toward A (see Fig. 25). Because of the changes in the lungs the body cannot successfully eliminate the excess of carbon dioxide by hyperpnea. However, in response to the increased tension of carbon dioxide, the alkaline reserve of the body gradually rises and the kidney decreases the elimination of sodium. The point A is therefore raised toward A', and in this manner the pH is brought back toward normal. First the numerator of the fraction $\frac{H_2CO_3}{BHCO_3}$ is raised by the pathologic process in the lungs. As a compensatory response, base is retained in the body, the bicarbonate therefore increases, and the pH is thus altered but little.

Altitude.—Another example illustrating the integrated response of the body may be sought in the adaptations to altitude. At high altitudes the partial pressure of oxygen diminishes; for example, oxygen tension is decreased 50 per cent at four miles above the sea level. On a high mountain the body therefore receives less oxygen and at a lower partial pressure. This is the primary stimulus in the production of a definite chain of symptoms referable in part to the central nervous system; first, exaltation, then depression and poor judgment. Prominent is the dyspnea even on slight exertion. Among the responses to the diminished oxygen tension are a greater number of red blood cells and an increase in the circulation rate. These changes facilitate the carriage of a greater oxygen supply to the cells of the body in a given interval.

At high altitudes a type of breathing which is regularly irregular is

frequently noted. It is called, after those who described it, "Cheyne-Stokes breathing" and consists of alternate periods of hyperpnea and apnea. Due to oxygen lack, respiration is stimulated. This increases the concentration of oxygen in the alveoli, but carbon dioxide is simultaneously pumped out and then respiration is secondarily diminished. A period of apnea ensues which is finally terminated by oxygen-lack and especially carbon dioxide excess. Then the cycle of increased and diminished respiration is renewed. Such breathing may occur spontaneously in the two extremes of life and may be produced voluntarily as a result of overbreathing. It is an indication of a grave condition when associated with cardiac or nephritic disease. Here the underlying mechanism is one of lack of oxygen, since the inhalation of increased concentrations of oxygen usually restores the normal type of respiration.

Biot breathing is not to be confused with the Cheyne-Stokes type of respiration, for the former is totally irregular. It occurs during tubercular meningitis and is characterized by groups of two or three deep, rapid respirations followed by prolonged apneic periods.

The hyperpnea occurring at high altitudes is not always followed by a period of apnea, *i. e.*, Cheyne-Stokes respiration. However, it is constantly associated with a decrease of carbon dioxide tension and a resulting alkalosis. N is thus moved over to B (see Fig. 25). The alkalosis tends to decrease, though not to abolish the hyperpnea. As a result of the diminished hyperpnea, the alveolar oxygen tension continues raised though to a lesser degree, and that of carbon dioxide diminished to a lesser extent than was the case during the initially greater hyperpnea. The partial recovery of carbon dioxide tension permits the *pH* to return from B part way toward the normal value at P. In the adaptations to altitude, the action of another organ, the kidney, is brought into play. The kidney excretes increased amounts of sodium and less of ammonia. The effect of this renal adaptation is to diminish the height of the carbon dioxide dissociation curve from P toward E and at the same time bring the *pH* toward 7.4. After these changes have been effected, the subject is said to be acclimatized and the initial distressing symptoms are alleviated. Apparently the tissues become adapted to a lower oxygen tension. The circulation rate is diminished, but the increased number of red blood corpuscles is maintained. The amount of hemoglobin in each cell, however, is decreased since the total amount of hemoglobin in the blood is not increased at altitudes.³³

Diabetes.—During diabetes the body must make adjustments for the best possible compromise in the midst of several grave disturbances. In the first place, carbohydrate ceases to be oxidized, and this failure is the initial cause of the other changes. Since sugar cannot be burned, fats are oxidized in a normal manner only to the four carbon stage and then accumulate as the acetone substances, β -oxybutyric acid, aceto-acetic acid and acetone (column 2, Fig. 26). These acids neutralize bicarbonate, and produce an acidosis. The effect of acidosis is

to cause an overbreathing, more carbon dioxide is eliminated and the pH rises toward the normal value (column 2). With profound acidosis the hyperpnea becomes intense and the respiration assumes a deep stertorous sighing character called "Kussmaul breathing." At the same time, the kidneys are forming more ammonia and excreting a part of the ketone acids as ammonia salts. If, however, the formation of the acetone substances is rapid, then a larger portion will be excreted as sodium salts (column 3). As a result, the electrolyte level of the blood

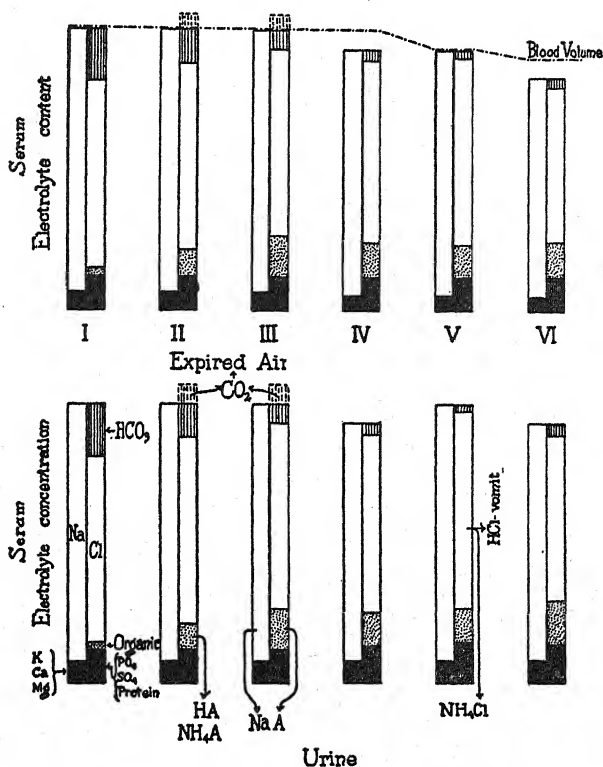


Fig. 26.—A schematic representation of the acid-base balance of serum during the development of diabetic acidosis. (J. P. Peters in *Yale J. Biol. Med.*, 2, 1930.)

diminishes (column 4). To keep the osmotic pressure constant, more water is eliminated raising the concentration of the serum as seen in column 5. Further loss of base and chloride finally brings about a diminished concentration of electrolytes despite the decrease in blood volume.

The effective therapy consists of the administration of insulin and glucose, water and salt. With this treatment the progressive series of changes of diabetes may be reversed, for the oxidation of carbohydrate and acetone substances is assured and renal function facilitated. The

osmotic and electrolyte equilibria, normal water balance and pH are restored, thus reestablishing the physiologic patterns of the body.

Summary.—In this discussion the exchange of gases in the blood has been described; first the mechanisms involved in normal quiet respiration, and then the series of adaptations brought into play to maintain the constancy of the internal environment when the body is subjected to unusual strain. It has been pointed out that in the transfer of oxygen and carbon dioxide both physical and chemical factors are involved and that the reciprocal actions of oxygen and carbon dioxide during the respiratory cycle are made possible by the mutually antagonistic effects of oxyhemoglobin and carbhemoglobin upon each other and the competition of carbon dioxide with oxyhemoglobin for base. As blood traverses the lymph it may liberate 4 volumes per cent of CO_2 and gain 5 volumes per cent of O_2 ; which is the reverse of what occurs in the tissue capillaries. The most important substance in these processes is the versatile compound hemoglobin, which carries not only almost all the oxygen exchanged as oxyhemoglobin, but also 60 per cent of the CO_2 (2.4 volumes per cent) by virtue of the base released by hemoglobin, and 33 per cent of the CO_2 (1.3 volumes per cent) as carbhemoglobin. Though carbhemoglobin may retain but 5–10 per cent of the total CO_2 content of the blood, it nevertheless assumes importance as a CO_2 carrier. The reversible combination with hemoglobin takes place with carbon dioxide in the anhydrous form; however that with base occurs through the intermediary carbonic acid and the reaction $\text{CO}_2 + \text{H}_2\text{O} = \text{H}_2\text{CO}_3$ is catalyzed by carbonic anhydrase.

The various types of acidosis and alkalosis have been considered, *i. e.*, those due to variations of the concentrations of carbon dioxide, and of fixed acids and of bases; and it has been pointed out that not only the blood but also the various organs are involved in the maintenance of the acid-base equilibrium. The functions of the liver, brain, heart, skin and blood have been considered in this regard. The lung and kidney are perhaps the two organs chiefly concerned with the regulation of the reaction of the body. The adaptations affected by the kidney can take place only with ample supplies of water in the body, for the kidneys tend to maintain the normal pH by the excretion respectively of fixed acid or base. The lung, on the other hand, regulates the acid-base equilibrium by controlling the elimination of the volatile acid metabolite carbon dioxide and does so more-over without loss of base.

Though the respiratory centers are acutely sensitive to changes in the concentrations of hydrogen ion and carbon dioxide, the characteristics of the arterial blood going to the respiratory centers constitute only some of their chemical influences. The concentrations of these acid respiratory stimulants are also affected by the carbon dioxide produced by the intrinsic metabolism of the centers and the rate of blood flow through the centers. In summary, it may be said that the

processes which tend to maintain a constant reaction of the body include not only the buffer actions of the blood, but also the excretion or retention of either acid or base, as well as the production of increased quantities of acid (lactic acid and ketone substances) or base (ammonia). The response of the body to a change of pH is not an isolated one; for a number of equilibria in addition to that of the hydrogen ion concentration, *i. e.*, carbon dioxide, oxygen, temperature, water, the various inorganic ions, are involved; and a change of any one of these equilibria may finally affect all the others. The bodily response is therefore a resultant, an integration; it may be regarded as a compromise. Examples of such working compromises are described in the adaptations to altitude and the changes occurring in emphysema and diabetes.

HAROLD E. HIMWICH.

REFERENCES

1. Henriques, O. M.: *Biochem. Z.*, **200**, 1, 5, 18, 22 (1928); **243**, 241 (1931); **260**, 58 (1933).
2. Margaria, R., and Green, Arda A.: *J. Biol. Chem.*, **102**, 611 (1933).
3. Himwich, H. E., and Barr, D. P.: *J. Biol. Chem.*, **57**, 361 (1923).
4. Van Slyke, D. D.: *Physiol. Rev.*, **1**, 141 (1921).
5. Christiansen, J., Douglas, C. G., and Haldane, J. S.: *J. Physiol.*, **48**, 244 (1914).
6. Meldrum, N. U., and Roughton, F. J. W.: *J. Physiol.*, **80**, 113, 143 (1933).
7. Stadie, W. C., and O'Brien, Helen: *J. Biol. Chem.*, **103**, 521 (1933).
8. Van Slyke, D. D., Wu, H., and McLean, F. C.: *J. Biol. Chem.*, **56**, 765 (1923).
9. Van Slyke, D. D., Hastings, A. B., Murray, C. D., and Sendroy, J., Jr.: *J. Biol. Chem.*, **65**, 701 (1925).
10. DuBois, D.: *Science*, **76**, 441 (1932).
11. Schmidt, C. F.: *Am. J. Physiol.*, **84**, 242 (1928).
12. Barr, D. P., and Himwich, H. E.: *J. Biol. Chem.*, **55**, 539 (1923).
13. Gesell, R.: *Physiol. Rev.*, **5**, 551 (1925).
14. Henderson, L. J.: *Am. J. Physiol.*, **21**, 427 (1908).
15. Nash, T. P., and Benedict, S. R.: *J. Biol. Chem.*, **48**, 463 (1921).
16. Hewlett, A. V., Barnett, G. D., and Lewis, J. K.: *J. Clin. Invest.*, **3**, 317 (1926-27).
17. Wilson, D. W., Long, W. K., Thompson, H. C., and Thurlow, S.: *J. Biol. Chem.*, **65**, 755 (1925).
18. Himwich, H. E., Koskoff, Y. D., and Nahum, L. H.: *J. Biol. Chem.*, **85**, 571 (1930).
19. Himwich, H. E., Koskoff, Y. D., and Nahum, L. H.: *Proc. Soc. Exper. Biol. Med.*, **25**, 347 (1928).
20. McGinty, D. A.: *Am. J. Physiol.*, **98**, 244 (1931).
21. McGinty, D. A.: *Am. J. Physiol.*, **88**, 312 (1929).
22. Himwich, H. E., and Nahum, L. H.: *Am. J. Physiol.*, **90**, 680 (1929); **101**, 446 (1932).
23. Barr, D. P., and Himwich, H. E.: *J. Biol. Chem.*, **55**, 525 (1923).
24. Snapper, I., and Grünbaum, A.: *Biochem. Z.*, **206**, 319 (1929).
25. Fishberg, Ella H., and Bierman, W.: *J. Biol. Chem.*, **97**, 433 (1932).
26. McClure, G. S.: *Am. J. Physiol.*, **99**, 365 (1932).
27. Macleod, J. J. R.: *Am. J. Physiol.*, **55**, 184 (1921).
28. Cook, L. C., and Hurst, R. H.: *J. Physiol.*, **79**, 443 (1933).
29. Evans, C. L., Graff, A. C. De, Kosaka, T., MacKenzie, K., Murphy, G. E., Vacek, T., Williams, D. H., and Young, F. G.: *J. Physiol.*, **80**, 21 (1933).
30. Mellanby, J., and Thomas, C. J.: *J. Physiol.*, **54**, 178 (1920).
31. Evans, C. L.: *J. Physiol.*, **56**, 146 (1922).
32. Haldane, J. B. S., Wigglesworth, V. B., and Woodrow, C. E.: *Proc. Roy. Soc. (London)*, **96B**, 25 (1924).
33. Hurado, A.: *Am. J. Physiol.*, **100**, 487 (1932).
34. Peters, J. P.: *Yale J. Biol. Med.*, **2**, 183 (1930).

CHAPTER XVII

RESPIRATION AND RESPIRATORY METABOLISM

WITHOUT respiration life cannot continue even for a matter of minutes, for the continuous interchange of respiratory gases is essential to vital processes. Perhaps the most important object accomplished by breathing is the acquisition of oxygen, although the elimination of carbon dioxide is equally necessary. Carbon dioxide acts as an acid within the body, and therefore when it is given off in the expired air, it affects the acid-base equilibria of the body and the pH of the blood. Expired air, furthermore, is saturated with moisture. The air comes from the lungs, where it has been in contact with the alveolar membrane, and the capillaries of this membrane permit the diffusion of the water of the blood coursing through them. Thus, the expired air contains water vapor and the evaporation of this moisture requires heat. In this manner the loss of moisture in the expired air affects the temperature of the body. We see, therefore, that the oxygen and carbon dioxide exchange of the body, its pH and temperature, are all regulated in the process of breathing. Since the body acts in such a manner as to maintain these four factors constant, any variation of one or more of them may cause a change of respiration in an effort to restore their normal relations. Because of this respiratory response oxygen, carbon dioxide, pH and temperature are stimuli of respiration.

The advantage derived from the acquisition of oxygen is obtained when oxygen combines with foodstuffs. During the processes of oxidation energy is liberated, and with this energy the cells are enabled to live and work. In the final analysis, the energy necessary to perform the work of the body comes from oxidations. The contraction of muscle, the secretion of glands, the conduction of nerve, in truth, are complicated processes. However, when the long chain of events takes place which yields the energy necessary for the functions of the various parts of the body, the final link consists of an oxidation.

In systems which are not biogological, but artificially constructed by man, as is the steam engine, for example, oxygen can combine with a substance like coal only when the process is catalyzed by a high temperature. In the living body, however, the combination of oxygen with the proximate principles, *i. e.*, the end-products of the digestion of the foodstuffs, occurs at a much lower temperature. The temperature of mammals, for example, is close to 37° C. In place of the high temperature of the coal furnace, the body possesses enzymes which catalyze the processes of oxidation. Carbon, hydrogen and nitrogen are the chief constituents of foodstuffs. After combination

with oxygen, carbon dioxide and water are formed. The nitrogenous foodstuffs are not directly oxidized. First, the nitrogen is removed from the amino acids by deamination. The remainders may then be oxidized to carbon dioxide and water.

A definite relationship exists between the oxygen utilized and the carbon dioxide produced. When carbohydrates are oxidized, the volumes of carbon dioxide and oxygen are equal, yielding a *respiratory quotient* of unity: $\frac{\text{vol. CO}_2 \text{ produced}}{\text{vol. O}_2 \text{ consumed}} = 1$. Fat contains more hydrogen than does carbohydrate, therefore additional oxygen is required for the formation of water. Thus a greater volume of oxygen is consumed than is used for the formation of carbon dioxide and the ratio between the volumes of carbon dioxide and oxygen is less than unity. It is actually 0.7.

Simple unicellular animals are able to perform all the fundamental processes necessary to life. As the protozoan floats in its liquid environment, it absorbs oxygen and gives off carbon dioxide. In multicellular animals, most of the cells are situated deep within the body and not in contact with the air. Survival of such complex animals must therefore depend upon the development of an interior environment which facilitates the exchange of gases which is so fundamental to life.

Such an environment is offered by the blood, the *milieu interieur* of Claude Bernard. The blood takes the place of the outside environment of the unicellular animal and facilitates cellular respiration. This is the respiration truly vital to life. Another name for *cellular respiration*, which distinguishes it from other portions of the mechanism of respiration, is *internal respiration*. The processes which take place in the lungs, whereby the blood gives off the carbon dioxide acquired from the cells and takes in additional amounts of oxygen destined for the use of the cells, may, in contrast, be called *external respiration*. In such a process, with diffusion of gases through the lungs at one end of the chain of events and diffusion of gases through the cellular membranes at the other end, the blood acts as the intermediary. It plays the part of the middleman.

External Respiration.—External respiration takes place in the lungs which are enclosed in the chest cavity bounded by the diaphragm below and the ribs on the sides and above. The lungs are connected externally with the air by a large tube, the windpipe or trachea. The trachea divides into the two primary bronchi, one to each lung. These bronchi continue dividing and subdividing until they become the very narrow tubes leading to the terminal air sacs or alveoli. The alveolus is surrounded by the pulmonary capillaries. The blood thus circulates around each alveolus and since the alveolar walls are thin, the gases diffuse through them.

Mechanism of External Respiration.—There is a great advantage in this enormous subdivision of the pulmonary surface, increasing as it

does the area available for *diffusion*. The surface area of the lungs is not large, perhaps three square feet; the surface area of the alveoli is tremendous, approximately 1000 square feet. After the mixed venous blood leaves the right ventricle and enters the lungs through the pulmonary arteries, these also divide until they become the pulmonary capillaries. As a result of this subdivision of the vessels containing the blood, the blood itself is divided from the original single large stream into many small ones. In this manner an enlarged area of blood is exposed to the processes of diffusion. Because of the large expanse of surface of alveoli and blood, the gaseous exchange taking place in the lungs in a unit of time is greatly enhanced.

In Fig. 27 on the inside of the thin alveolar membrane is seen a capillary and on the outside, air. The gases, oxygen and carbon dioxide, diffuse according to their pressure gradients. The pressure of

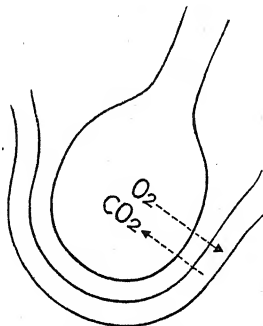


Fig. 27.—Schematic representation of capillary and alveolus.

oxygen is greater in the alveolar air, therefore oxygen diffuses into the blood; while the pressure of carbon dioxide is greater in the blood, causing carbon dioxide to diffuse outward to the alveolar air. In a similar fashion, the oxygenated blood supplied to the body in the arteries has a higher tension or pressure of oxygen, and a lower pressure of carbon dioxide than have the tissues. The tissues, in their turn, contain carbon dioxide at a slightly higher tension than it exists in the venous blood. The tension of oxygen in the various tissues of the body is not the same. In some parts of the body it may approach that of the venous blood leaving the tissues, and this pressure may be high in the case of glands (such as the kidney or thyroid). In other tissues, for example, resting muscle, though the capillaries are continually closing and opening, at any given time most of the capillaries are closed so that the blood supply is limited and the oxygen tension existing in muscle approaches zero.

Oxygen.—It may be helpful to discuss the actual tensions of oxygen as they obtain in the several parts of the body (Fig. 28). The inspired air contains one fifth of an atmosphere of oxygen which is therefore at a pressure of 150 mm. Hg at sea level. Alveolar air has approxi-

mately one eighth of an atmosphere of oxygen, about 100 mm. Hg tension. It may be expected that alveolar oxygen tension should be intermediate between that of inspired air, and that of mixed venous blood, the two extremes concerned in pulmonary ventilation. Arterialized blood holds oxygen at a tension of 80 mm. Hg. Apparently a head of pressure of 20 mm. Hg is required to force oxygen across the alveolar membrane. The necessary oxygen supply in this manner enters the blood and is then transported to the points where it will be ultimately utilized. Thus, once the blood leaves the lungs, it is pumped by the left heart into the arteries and finally enters the capillaries. From the capillaries oxygen diffuses into the various parts of the body which absorb oxygen according to their respective require-

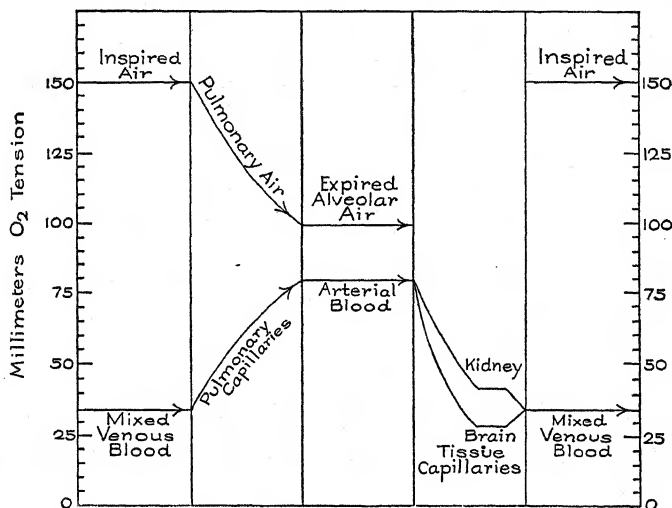


Fig. 28.—Changes in O₂ tension of air and blood. (Adapted from Peters and van Slyke.)¹⁷

ments; for example, the brain removes more oxygen per 100 cc. of blood passing through it than does the kidney.

The continuous utilization of oxygen by the tissues affords the difference of pressure required to force the oxygen, which is so necessary to life, across the capillary wall and cellular membranes into the cell. Oxygen continues to enter the tissues until the difference of pressure is no longer sufficient to facilitate diffusion. This process reduces the oxygen tension of the blood and the venous blood returning from the tissues must then contain oxygen at a tension lower than that of the arterial blood. The statistical average of the oxygen tensions of the venous blood of the entire body during rest is found in the right heart at 35 mm. Hg.

Carbon Dioxide.—In the case of carbon dioxide, in contrast with oxygen, a gas is eliminated instead of absorbed. Inspired air contains

carbon dioxide at a tension of 0.2 mm. Hg, the alveoli at 40 mm. Hg. The arterial blood retains carbon dioxide at a partial pressure close to 40 mm. Hg since the great solubility of carbon dioxide necessitates but little pressure difference to force it across the alveolar membrane. The tissues, the source of the carbon dioxide, contain carbon dioxide at its highest tension in the body. The blood therefore receives an increment of carbon dioxide as it passes through the capillaries and the statistical average of the carbon dioxide tensions of the mixed venous blood of the body during rest is 46 mm. Hg.

Respiratory Movements.—Though the property of diffusion of gases and the large surface provided for diffusion aid the exchange of gases through the alveolar membrane, the volume of the gaseous exchange

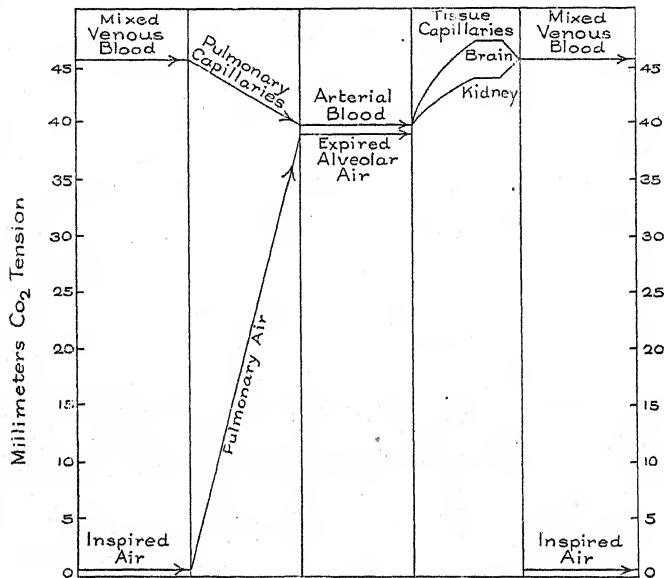


Fig. 29.—Changes in CO_2 tension of air and blood.

facilitated by these mechanisms, if unaided by the respiratory movements, is insufficient to maintain life. The pumping of air into and out of the lungs is necessary to maintain the comparatively high oxygen tension and low carbon dioxide tension which exists in the alveolar air. If the movements of respiration were stopped even with all the air passages wide open, it would be impossible to maintain life. In a disease called "emphysema," there is a loss of elastic tissue of the lungs and a diminution of the respiratory movements. As a result, the oxygen content of the blood diminishes and there may be, in severe cases, a retention of carbon dioxide. Since the pumping movements of the thoracic wall are of the utmost importance, it is of interest to see in what manner they aid in the entrance and exit of the pulmonary air.

It is not sufficient for the thoracic walls to enlarge the thoracic cavity; the lungs within the thorax must also increase in capacity. The lungs, though not attached to the thoracic wall, nevertheless follow them in their movements because of the suction exerted by the negative pressure of the thoracic cage. This suction is made possible by the presence of the two layers of pleurae. The lungs are divided into large divisions or lobes and each lobe is surrounded by a membrane, a sheath, the visceral pleura formed of flat mesothelial cells and connective tissue. The sheath leaves the lung at the root or hilum, at which place it is continued on to the thoracic wall and diaphragm. A cross section of the thorax reveals first the thoracic wall, then, in succession, a layer of parietal pleura, a potential cavity, and finally the visceral pleura on the lung tissue itself.

The two smooth layers of pleurae glide on one another aided by a small amount of lubricating intrapleural fluid. When the lungs are pulled away from the thoracic wall, a negative pressure is formed between the two layers of the pleurae. Opposing this expansile influence is the elastic tissue of the lungs. The elastic tissue constantly tends to cause the lungs to collapse; and this would occur were it not for the negative pressure between the pleurae which counteracts the elastic pull and causes the lungs to expand. The position of the lungs in the thoracic cavity is therefore the resultant of the pull of the elastic tissue elements of the lungs and the negative pressure between the two pleural layers. During inspiration, the thoracic wall enlarges and the pressure between the pleurae is decreased. The pressure during quiet inspiration may fall from an initial value of -5 mm. Hg to -10 mm. Hg. The greater extrapulmonary suction causes the expansion of the lungs. The intrapulmonary pressure is thus reduced and air rushes in through the trachea and smaller air tubes. During expiration, the thoracic cage becomes smaller in volume, the pressure between the two layers of the pleurae increases from -10 to -5 mm. Hg. As a result of the diminished extrapulmonary suction, the elastic tissue of the lungs is permitted to contract. The intrapulmonary pressure increases, and air is forced out through the various air passages.

A sharp distinction should be made between the intrapulmonary pressure and the extrapulmonary or intrathoracic pressure. In quiet respiration, the intrapulmonary pressure never varies very much from that of the outside air, either when positive in expiration or negative during inspiration. The extrapulmonary pressure, in contrast, is always negative. It is obvious that if the negative pulmonary pressure were to be abolished, the lungs would collapse entirely simply as a result of their own elastic recoil. This procedure is employed in the treatment of conditions in which the collapse of one lung is indicated; for example, in lobar pneumonia and pulmonary tuberculosis confined largely to one lung. In such a case, nitrogen is often injected in the intrapleural space. Thus, the negative pressure is abolished and the lung remains collapsed until the nitrogen is absorbed by the blood.

The rest afforded the collapsed lung is favorable for the healing of its affected parts.

The movements of the thoracic wall are the primary cause of the exchange of air between the lungs and the outside environment. The inspiratory movements are performed as a result of the contraction of various muscles. Expiration, in contrast with inspiration, is not an active process but largely a passive one following the relaxation of muscles, the gravitational fall of the ribs and the elastic rebound of the lungs.

Inspiration is brought about by an increase in all the diameters of the chest. The vertical diameter is increased by the descent of the diaphragm, a thin sheet of muscle with a central tendon separating the chest from the abdomen. The diaphragm is innervated by the phrenic nerve. When impulses are sent down the phrenic nerve from the respiratory centers in the medulla and pons the diaphragm contracts. In expiration the diaphragm appears as a double cupola. During inspiration it is lowered and straightened out by the shortening of its muscle fibers. The diaphragm is pulled away from the ribs, thus opening the pleural sinuses into which the lungs descend. In this con-

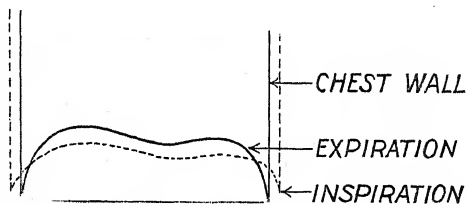


Fig. 30.—Positions of diaphragm during expiration and inspiration.

traction the diaphragm presses on the contents of the abdomen so that the abdomen swells with each inspiration. This action is aided by the reciprocal innervation of the abdominal muscles and the diaphragm, which permits the relaxation or loss of tone of the abdominal muscles; while the diaphragm contracts and the regaining of the tone of the abdominal muscles when the diaphragm relaxes.

The movements of the ribs also enlarge the chest cavity. Impulses originating in the respiratory centers are carried by the intercostal nerves to the external intercostal muscles and the serratus posterior superior which is attached to the second, third, fourth, and fifth ribs. The movements of the ribs change all the diameters of the thorax. In the first place the ribs are hung downward at an acute angle to the spinal column so that when they are raised the anterior-posterior diameter is enlarged. Secondly, the first seven pairs of ribs form rings which increase in circumference from above downward. When the ribs are raised in inspiration each pair taking the place of the one above forms a ring which is larger not only anterior-posteriorly but also from side to side. Finally, the ribs are bowed and during expiration the bowing is outward and downward. During inspiration the

ribs are rotated so that their position is less in a downward and more in an outward direction. In this manner the lateral diameter of the thorax is enlarged.

Though normal expiration is largely passive, forced expiration brings into play many groups of muscles. The abdominal muscles contract in order to aid the ascent of the diaphragm. The contraction of the internal intercostal muscles lowers the ribs; and that of the serratus posterior-inferior has a similar effect on the last four pairs of ribs. During forced respiration accessory muscles are active, muscles of the face, neck, chest and abdomen. In order to comprehend the effects of forced respiration it is necessary first to consider the normal respiratory volume. In normal quiet inspiration and expiration of a man weighing about 70 kilos, approximately 500 cc. of air enter and leave the lungs. This volume is called the "tidal air." If, after a normal inspiration, a forced inspiration is made, an additional 2000 cc. of air may be taken into the lungs—the "complemental air." Similarly, after a normal expiration, it is possible to force out an added 1500 cc. of air—the "supplemental air." Complemental, tidal and supplemental air together form "vital capacity." This is obtained by collecting the largest possible expiration after the greatest possible inspiration; and in a man of average weight, it is approximately 4000 cc. "Vital capacity" is larger in bigger men and also in athletes. It is smaller in women.

Even after a forced expiration there still remains in the lungs another 1500 cc., the residual air. Thus, after quiet expiration the lungs contain 3000 cc. of air, *i. e.*, supplemental and residual. It should be noted that of the total 500 cc. of tidal air, only 350 cc. reach the alveoli. Approximately 150 cc. remain in the larger air passages. These constitute the "dead space." As a result 350 cc. of incoming air is diluted by 3000 cc. already in the lungs. The first part of expired air may be indistinguishable from outside air. Therefore, there is a gradient of oxygen and carbon dioxide pressures between atmospheric and alveolar air. It is only the last portion, technically, the last 100 cc. which has certainly come from the alveoli and is therefore called alveolar air. Whenever quiet respiration is increased, for example, in the present illustration when the tidal air is more than 500 cc. the type of breathing is referred to as *hyperpnea*. During hyperpnea a larger volume of incoming air dilutes the pulmonary air thus raising the content of oxygen and diminishing that of carbon dioxide in the alveoli. Hyperpnea always occurs during physical exertion since it is necessary to maintain the greater gaseous exchange between alveolar air and blood. Increased breathing may also be observed in certain diseases of the lungs and heart. If hyperpnea continues for some time breathing becomes painful and is called *dyspnea*. The terms hyperpnea and dyspnea are sometimes used synonymously; but breathing may be very painful, as in pleurisy, without any hyperpnea; indeed, there may even be a limitation of the respiratory movements.

Hyperpnea and dyspnea are most frequently the result of a diminished vital capacity. In patients suffering from lobar pneumonia or cardiac disease with pleural effusion, the vital capacity is decreased and at the same time a smaller alveolar surface is exposed to diffusion. The forced breathing is therefore a compensatory action to increase the exchange of gases between the alveolar air and the blood. In emphysema the chief cause of the diminished vital capacity is an enlarged residual air due to the inability of the lungs to contract. The incoming air is therefore diluted by a larger volume of pulmonary air than in the normal individual, and the patient suffers a diminished gaseous exchange. An increased tidal air, however, tends to compensate for the larger volume of residual air. Vital capacity is therefore of clinical interest and the changes in the physical condition of the patient may be paralleled by those of vital capacity.² A decreased vital capacity limits the reserve of the lungs; *i. e.*, it lessens complementary and supplemental air and therefore permits only a smaller increase of breathing during exertion. Finally, with a great diminution of vital capacity dyspnea will occur even when the patient is lying in bed.

Orthopnea is the name given to the type of breathing that is made easier by sitting upright. This is noted characteristically in patients with severe cardiac disease who must remain in a sitting position throughout the twenty-four hours of the day. There are several advantages to be gained by assuming this position instead of lying flat. The brain, and therefore the respiratory centers, are drained more easily of venous blood. The shoulders may be fixed better and thus permit greater play of the muscles of respiration. Perhaps most important is the greater vital capacity. Not only is the diaphragm in a lower position but the pooling of blood in the thoracic cavity which occurs when lying, disappears in assuming the upright position (Hamilton and Morgan³).

Regulation.—It is difficult to conceive of such a complicated process as breathing without centers in the brain to coordinate and regulate the various component processes, so that they may function together as a unified whole. Bilateral centers probably exist in the brain stem, the pons and medulla. If the upper portions of the brain, the cerebrum and the cerebellum, are removed, respiration may still continue; but a section below the medulla will promptly stop all spontaneous breathing. Experiments on lower animals, *i. e.*, fish, prove that the respiratory centers are automatic, periodically sending out motor discharges.⁴ Probably in the higher animals the respiratory centers are also automatic. This automaticity, however, is keenly sensitive to afferent stimulation; so that breathing may be accelerated or temporarily stopped.

Afferent stimulation to the centers may come through nerve paths. Perhaps the most important one is the vagus. When the vagus is sectioned, respiration is slowed. The function of the intact vagus is

therefore to increase the respiratory rate. This function is performed because of the inhibitory effects of the vagus nerve on the amplitude of respiration. As a result of the smaller inspiratory volume, the following expiration is shorter too. Breathing is thus accelerated. The vagus carries impulses arising in pulmonary end-organs which respond chiefly to stretching of the pulmonary tissues. With each inspiration, these end-organs send stimuli through the vagus to the respiratory centers—stimuli, which tend to inhibit the further progress of inspiration.⁴

The trigeminal and glossopharyngeal nerves are of lesser importance. The former is protective in action; for it carries the impulses which arise in the nose as a result of irritation of its mucous membrane, and therefore initiates sneezing or a temporary stoppage of breathing. The latter nerve assists in the coordination of breathing that must occur during swallowing.

The respiratory centers are sensitive to changes in all parts of the body. Deep sensations from the abdominal and thoracic walls are of importance. These reach the cerebrum partly by way of the vagi and partly by the dorsal roots of the spinal nerves. Among other phenomena, the impulses carried by these nerve paths may explain the cessation of diaphragmatic breathing occurring during acute abdominal inflammation or peritonitis. Hiccough is also a vagal effect. The respiratory centers react to stimuli arising from the skin. These, too, may be relayed *via* the cerebrum; as for example, in the change of breathing which occurs while taking a cold shower. Respiration, within certain limitations, may be controlled voluntarily. Emotional states, fear or anger, also affects respiration; hyperpnea begins even before the race starts.

End-organs in the arch of the aorta and in the carotid sinus also send nervous impulses to the respiratory centers through the vagus and glossopharyngeal nerves. An increase of blood pressure is followed by a diminished rate of respiration and *vice versa*.⁵ There are probably other end-organs in the arch of the aorta and the carotid sinus which are sensitive to a diminished oxygen content of the blood and cause a compensatory increase of respiration by their influence on the respiratory centers.⁶ The respiratory centers, on the other hand, react directly without nervous intermediary to changes of carbon dioxide and *pH* of the blood.

The chief stimuli of the respiratory centers are disclosed in experiments performed by Heymans and Heymans.⁷ The head of dog *B* received its blood supply from dog *A* and was entirely isolated from the trunk of dog *B* with the exception of its vagal connections. Under such conditions the isolated head of dog *B* continued to make automatic respiratory movements which were registered by means of the apparatus 8. These movements, nevertheless, were greatly influenced by stimuli coming to its respiratory centers in its own vagus nerves, as well as in the blood of dog *A*. The effect of the position of the

lungs was apparent when the artificial respiration of trunk *B* was stopped so that the lungs were in maximal inspiration. In such a condition, the centers attempting to initiate the next respiratory movements finally caused the breathing of the isolated head of dog *B* to cease in an expiratory position. Similarly, if the artificial respiration of trunk *B* was stopped in the collapsed position of the lungs, the isolated head ceased breathing in an inspiratory position. The earlier effect, however, of asphyxia of the trunk was not to stop breathing, but rather to produce hyperpnea of its isolated head; no doubt, due, in part, to lack of oxygen in the blood—a reflex mediated by the end-

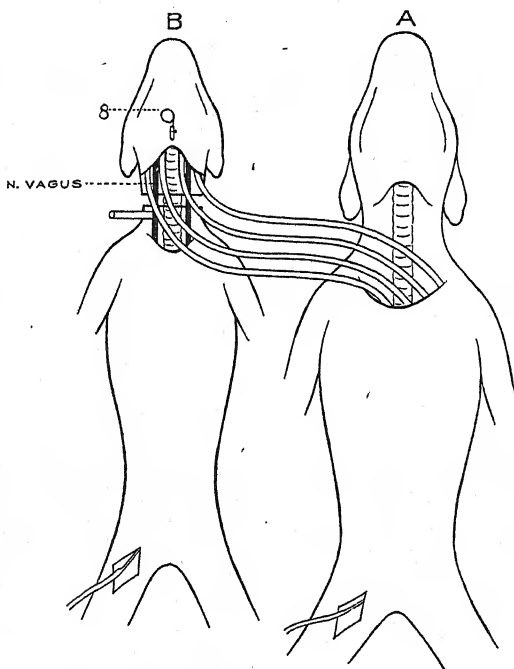


Fig. 31.—Method of differentiating between vagal and hematic respiratory stimuli (Heymans and Heymans').

organs of the carotid sinus. A rise in the blood pressure of trunk *B* resulted in apnea, while a fall in blood pressure increased the rate of breathing.

On the other hand, the intravenous injection of an acid, either lactic or hydrochloric, into dog *A*, or the inhalation of carbon dioxide by dog *A*, produced increased respiratory movements on the part of the isolated head of dog *B*. These stimuli could have been carried to the isolated head only in the blood.

These experiments are conclusive in demonstrating the sensitivity of the respiratory centers to the character of its profusing blood. How-

ever, of secondary importance in regard to the response to changes of carbon dioxide and pH of the blood, is the function of the carotid sinus. It has been shown that denervation of the carotid sinus reduces somewhat the reaction of an animal to increased carbon dioxide.⁸ This makes it probable that the centers are stimulated through the responses of the sinuses to the concentrations of carbon dioxide and pH of the blood. Similarly, it should be mentioned that although the chief effect of lack of oxygen is an increased breathing mediated by the carotid sinus, anoxemia exerts a direct and opposite effect on respiratory centers, depressant in character, as has been demonstrated in animals with denervated carotid sinuses.⁹ However, in the intact animal, only during profound anoxemia does the direct action of oxygen-lack upon the centers become the predominant one.

Summary.—In a summary of the processes of external respiration, it should be emphasized that the need of the cells is the cause of respiration; that the blood brings oxygen and removes carbon dioxide from the cells; and that the lungs perform a similar service for the blood. This is done because of the characteristic of diffusion possessed by the gases, the large alveolar surface and blood areas exposed to diffusion and the pumping respiratory movements which change the pulmonary air. These mechanisms facilitate the absorption of the large volumes of oxygen necessary for the maintenance of life in the warm-blooded animals.

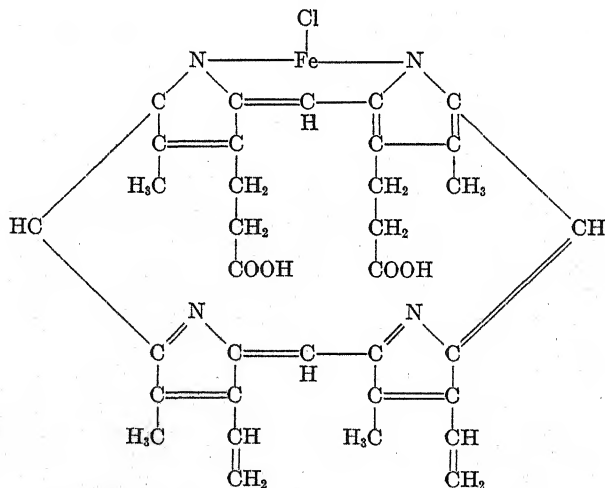
The entire phenomenon of respiration is controlled by centers which in response to stimuli chiefly mediated by the vagus (position of the lungs and oxygen-lack) and by the blood (carbon dioxide and pH) integrate the complicated series of respiratory processes so that they may act as a unit. These centers discharge motor impulses periodically; but the rate of discharge is usually modified by afferent stimulation. During inspiration, motor impulses are sent to the diaphragm and the external intercostal muscles which cause them to contract and enlarge the thoracic cavity. The extrapulmonary pressure is diminished, and the lungs are thus distended. The intrapulmonary pressure is, therefore, decreased, and air rushes into the lungs. During this time, afferent nervous and chemical stimuli act upon the respiratory centers tending to limit inspiration. As a result, inspiration is finally inhibited; motor impulses no longer go to the diaphragm and intercostal muscles; and these, therefore, relax. The diaphragm rises and the ribs fall to diminish the capacity of the thorax. The lungs are therefore permitted to collapse, due to the recoil of their own elastic tissue, and air is thus forced out.

CELLULAR RESPIRATION

The passage of oxygen has been followed from the air of the atmosphere through the alveoli and pulmonary capillaries to the blood, as well as from the blood through the tissue capillaries to the cells (Chapter XVI). The next series of mechanisms to be considered are those

by which the cells utilize this oxygen. Outside the cells, the proximate principles of the foodstuffs are not oxidized even though oxygen may be present. It is therefore apparent that various preparatory stages must intervene before oxygen can combine with the carbon and hydrogen of the foodstuffs and the energy necessary for the life and function of the cell can be liberated.

It has been known for some time that the presence of a heavy metal, probably iron, is necessary for cellular oxidations to take place. Since iron occurs only in small amounts in the cells, it cannot react stoichiometrically, but rather must do so in a catalytic manner. No free iron is present in the tissues. On the contrary, it is bound in the form of *heme*.¹⁰ This compound is also called *hematin* and is a constituent of many substances that are involved in the transfer of oxygen. Heme consists of four pyrrol nuclei bound with one atom of iron and its structural formula as a chloride (*hemin*) is presented below. When heme is united with a nitrogen-containing compound it is called a *hemochromogen*; and many, if not all, the heme constituents of tissues are hemochromogens. Another compound of heme is one with the colorless globin which goes to form the red hemoglobin. Heme



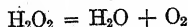
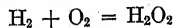
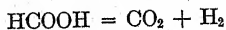
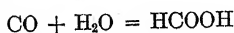
reacts reversibly with oxygen only when combined with native globin, forming an unknown linkage which, however, is not that of oxidation and reduction. Molecular oxygen is combined in the lungs with hemoglobin and given off in the tissues in the manner already described. Once the molecular oxygen enters the cells, it undergoes an entire series of transfers as a result of combining with respiratory ferments.

In contrast with the reversible reaction of oxygen with hemoglobin, the tissue hemes, as far as now known, are alternately oxidized and reduced, this reaction involving a change of valence of the iron atom. The first of the series of transfers is accomplished by a ferment de-

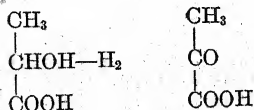
scribed by Warburg.¹² This ferment is inactivated by carbon monoxide or cyanide, which probably combines with the iron, thus preventing any further reaction with oxygen.

Next in the series are three other hemes, *cytochrome, a, b, and c*.¹¹ When studied spectroscopically in intact living cells these hemes in the reduced form reveal typical bands in the green portion of the spectrum—bands which disappear in the oxidized form. In the presence of cyanide or carbon monoxide, which stops the reaction of the respiratory ferment of Warburg with oxygen, the bands characteristic of the reduced form do not disappear. Apparently cytochrome can combine with oxygen only after that substance has been presented by Warburg's ferment. The mechanism of the poisonous action of carbon monoxide or cyanide is thus made clear. These poisonous substances stop the oxidations that are required for the flow of energy necessary to maintain life, because of their effect on Warburg's ferment. The chain of oxidations and reductions therefore stops at this point. Though carbon monoxide reacts with the respiratory ferment, it reacts even more readily with the heme of hemoglobin to form carboxyhemoglobin to the exclusion of oxyhemoglobin. Thus, in carbon monoxide poisoning, oxygen may not even enter the blood in significant amounts.*

However, the proximate principles, or, as we may now call them, substrates, are not ready to combine with the oxygen even after it has been transported *via* the four hemes. The substrate, too, must originally exist in a relatively inert form. It is therefore probable that this relatively inert form is changed in the cell in such a manner that it may be able to act as a reducing substance to the oxidized form of cytochrome *a*. Unfortunately, our knowledge of this part of oxidation is not so direct as that described above, but depends in larger part on analogy and *in vitro* reactions. Among the principal exponents of the view that a change in the stability of the oxidizable material is the cause of oxidations is Wieland.¹³ According to him, the mobilization of the hydrogen is the essential mechanism of oxidation. The process of oxidation of carbon monoxide, for example, may take place as follows:

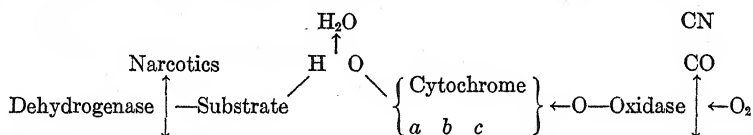


Wieland regards biochemical oxidations as essentially the catalytic transfer of hydrogen from the oxidizable material:



* For a more detailed discussion of the various pigments, see Chapter XVIII.

For example, from some lactic acid-forming bacteria, a heat-labile enzyme may be extracted which, in the absence of oxygen, may nevertheless oxidize lactic acid to pyruvic acid. This can be ascertained by the addition of methylene blue, which combines with the hydrogen mobilized by the enzyme and is therefore converted to the leuco form. On the addition of molecular oxygen, the leuco form is reconverted to oxidized methylene blue and, at the same time, yields two atoms of hydrogen for the formation of water. In the living cells (for example, of muscle), there are similarly acting heat-labile enzymes, *dehydrogenases*, which are capable of mobilizing the hydrogen of specific substrates and liberating it in atomic form. These enzymes thus act as hydrogen activators and permit the reduction of the oxygen transported by the hemes with the resulting formation of water.



The conception presented here is largely taken from Keilin² who brings together the two points of view; *i. e.*, of oxygen transfer (Warburg²), and of hydrogen mobilization (Wieland⁴) in a unified hypothesis. The two points of view are not necessarily contradictory but, on the contrary, complimentary in a form in which they have just been described. Oxidation and reduction are fundamentally transfers of electrons ($\text{Fe}^{++} - e \rightarrow \text{Fe}^{+++}$); so that whether oxygen is added or hydrogen is removed there is a loss of an electron. On the other hand, the process of reduction consists in the gain of an electron. Just as it has been found that carbon monoxide and cyanide prevent the oxidation of cytochrome, so its reduction is prevented by narcotics; for example, urethane or alcohol. Narcotics apparently interfere with the action of the dehydrogenase so that the enzyme can no longer mobilize the hydrogen of the substrate. In the absence of active or atomic hydrogen the reduction of cytochrome ceases.

The scheme here presented can account for the water formed during respiration. It can also explain the formation of carbon dioxide as described above. Nevertheless, enzymes other than those included in the scheme have been discovered. These other enzymes may, however, function in coordination with those described in the scheme. There are *oxidases*—hemes similar or perhaps the same as those of the respiratory ferment, which transport oxygen to substrates. *Peroxi-dases* have also been studied. These, which may be hemes, cause the liberation of oxygen from the peroxide formation. *Glutathione* is a tripeptide of cystine, glycine and glutamic acid. By virtue of the sulfur group ($-\text{S}-\text{S}-$) of the cystine constituent of two molecules of glutathione which may be reduced to $\text{H}-\text{S}-\text{S}-\text{H}$, glutathione acts as a hydrogen acceptor, but needs no heat-labile enzyme to mobilize

the hydrogen, nor does it require a specific substrate, since glutathione can accept the hydrogen of protein. Finally, mention may be made of a *carboxylase* which acts directly on the carboxyl group, and causes the release of carbon dioxide. This is another explanation of the carbon dioxide produced during respiration. It would be of interest to determine the relationship between carboxylase and the carbonic anhydrase of Meldrum and Roughton.¹⁴ *

BASAL METABOLISM

The mechanisms whereby the molecular oxygen of the atmosphere and the proximate principles of the foodstuffs are prepared for cellular oxidations have been described. Since the energy liberated during these oxidations is necessary for the maintenance of life, the measurement of this energy is a problem of major importance.

All forms of energy must finally be dissipated as heat. If an individual is placed in an adiabatic box, a calorimeter, and the heat eliminated from the subject by radiation, conduction and evaporation determined, a quantitative measurement of heat production may be made. The energetic equivalent of this heat, expressed as calories, is a measure of the energy used by the body. This method is that of *direct calorimetry*.¹⁵

The utilization of energy may also be measured indirectly by calculations based on the amount of urinary nitrogen and the volume of the gaseous exchange in the lungs. The results obtained by *indirect calorimetry* agree closely with those of direct calorimetry. Indirect calorimetry, however, yields additional information. Not only the total energy utilized by the body, but the contributions thereto by each of the foodstuffs, carbohydrate, fat and protein are estimated. The energy liberated per gram of foodstuff varies with its character; for carbohydrate it is 4.1 calories; for protein, 4.1 calories; and in the case of fat, 9.3 calories. The partition of the energy derived from the various foodstuffs is important, not only in the calculation of normal dietaries, but also in the study of disease; for example, in diabetes, with disturbances of carbohydrate and fat metabolism and in nephritis, with changes in protein metabolism.

Urinary Nitrogen.—On a diet of digestible foods, the nitrogen of the feces represents that of the bacteria and epithelial secretions and excretions of the intestinal mucosa. The amount of nitrogen lost in the perspiration and by the growth of hair and nails is such a small fraction of the total nitrogen excretion that it may be neglected. The urinary nitrogen is, on the contrary, chiefly in the form of urea and is a measure of the protein used to supply energy to the body. After deaminization of the amino acids, the ammonia goes to form urea, while the deaminized remainders of the amino acids are oxidized. Since meat protein has an average nitrogen content of 16 per cent, each gram of nitrogen in the urine represents the oxidation of 6.25 Gm. of protein. It has been found experimentally that in the oxidation of 6.25

* Compare this section with Chapter XIX.

Gm. of protein, 26.51 calories are liberated, 5.91 liters of oxygen are consumed and 4.76 liters of carbon dioxide produced.

Gaseous Exchange.—The volume of oxygen utilized in respiration must obviously be related to the energy liberated during oxidations; and by employing the proper factors, the energy requirements may be calculated from the oxygen consumption. The ratio of the volume of carbon dioxide produced to oxygen consumed, *i. e.*, the *respiratory quotient* (*R. Q.*), yields additional information as to the nature of the foodstuffs oxidized, since the *R. Q.* of carbohydrate is 1 and those of fat and protein are approximately 0.7 and 0.8 respectively.

ANALYSIS OF THE OXIDATION OF MIXTURES OF CARBOHYDRATE AND FAT

| R. Q. | Percentage of total oxygen consumed by | | Percentage of total heat produced by | | Calories per liter O ₂ . | |
|-------|--|-------------|--------------------------------------|-------------|-------------------------------------|-------------------|
| | Carbohy- drate. (1) | Fat. (2) | Carbohy- drate. (3) | Fat. (4) | Number. (5) | Logarithm. (6) |
| 0.707 | 0 | 100.0 | 0 | 100.0 | 4.686 | 0.67080 |
| 0.71 | 1.02 | 99.0 | 1.10 | 98.9 | 4.690 | 0.67114 |
| 0.72 | 4.44 | 95.6 | 4.76 | 95.2 | 4.702 | 0.67228 |
| 0.73 | 7.85 | 92.2 | 8.40 | 91.6 | 4.714 | 0.67342 |
| 0.74 | 11.3 | 88.7 | 12.0 | 88.0 | 4.727 | 0.67456 |
| 0.75 | 14.7 | 85.3 | 15.6 | 84.4 | 4.739 | 0.67569 |
| 0.76 | 18.1 | 81.9 | 19.2 | 80.8 | 4.751 | 0.67682 |
| 0.77 | 21.5 | 78.5 | 22.8 | 77.2 | 4.764 | 0.67794 |
| 0.78 | 24.9 | 75.1 | 26.3 | 73.7 | 4.776 | 0.67906 |
| 0.79 | 28.3 | 71.7 | 29.9 | 70.1 | 4.788 | 0.68018 |
| 0.80 | 31.7 | 68.3 | 33.4 | 66.6 | 4.801 | 0.68129 |
| 0.81 | 35.2 | 64.8 | 36.9 | 63.1 | 4.813 | 0.68241 |
| 0.82 | 38.6 | 61.4 | 40.3 | 59.7 | 4.825 | 0.68352 |
| 0.83 | 42.0 | 58.0 | 43.8 | 56.2 | 4.838 | 0.68463 |
| 0.84 | 45.4 | 54.6 | 47.2 | 52.8 | 4.850 | 0.68573 |
| 0.85 | 48.8 | 51.2 | 50.7 | 49.3 | 4.862 | 0.68683 |
| 0.86 | 52.2 | 47.8 | 54.1 | 45.9 | 4.875 | 0.68793 |
| 0.87 | 55.6 | 44.4 | 57.5 | 42.5 | 4.887 | 0.68903 |
| 0.88 | 59.0 | 41.0 | 60.8 | 39.2 | 4.899 | 0.69012 |
| 0.89 | 62.5 | 37.5 | 64.2 | 35.8 | 4.911 | 0.69121 |
| 0.90 | 65.9 | 34.1 | 67.5 | 32.5 | 4.924 | 0.69230 |
| 0.91 | 69.3 | 30.7 | 70.8 | 29.2 | 4.936 | 0.69339 |
| 0.92 | 72.7 | 27.3 | 74.1 | 25.9 | 4.948 | 0.69447 |
| 0.93 | 76.1 | 23.9 | 77.4 | 22.6 | 4.961 | 0.69555 |
| 0.94 | 79.5 | 20.5 | 80.7 | 19.3 | 4.973 | 0.69663 |
| 0.95 | 82.9 | 17.1 | 84.0 | 16.0 | 4.985 | 0.69770 |
| 0.96 | 86.3 | 13.7 | 87.2 | 12.8 | 4.998 | 0.69877 |
| 0.97 | 89.8 | 10.2 | 90.4 | 9.58 | 5.010 | 0.69984 |
| 0.98 | 93.2 | 6.83 | 93.6 | 6.37 | 5.022 | 0.70071 |
| 0.99 | 96.6 | 3.41 | 96.8 | 3.18 | 5.035 | 0.70197 |
| 1.00 | 100.0 | 0 | 100.0 | 0 | 5.047 | 0.70303 |

The gaseous exchange in any particular case must be determined by observation. This is done by analyzing expired air for oxygen and carbon dioxide either gravimetrically or volumetrically.^{16, 17} These

analyses yield information on the total utilization of all three foodstuffs. By subtracting the oxygen utilized and the carbon dioxide produced during the oxidation of protein from the total oxygen and carbon dioxide exchanged, the oxygen and carbon dioxide due to oxidation of nonprotein foodstuffs are obtained. With this information, the amounts of carbohydrate and fat oxidized may be calculated. The number of calories per liter of oxygen at each *R. Q.*, due to both nonprotein foodstuffs, are presented in column 5 of the accompanying table, and the partition of the calories between carbohydrate and fat in columns 3 and 4. The addition of the calories of nonprotein origin to those derived from the oxidation of protein gives the total caloric exchange of the body. This result acquires increased significance when compared with standard values; for then both physiologic and pathologic deviations from the standards may be detected.

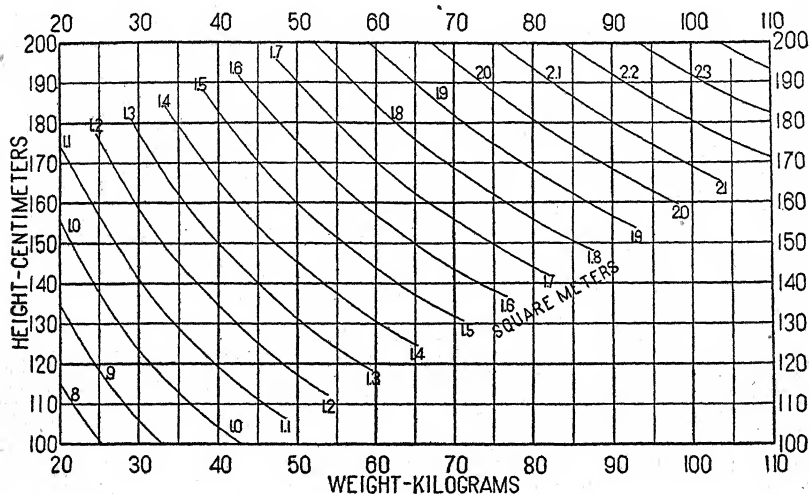


Fig. 32.—DuBois chart for determining surface area of man in square meters (area = $Wt^{0.425} \times Ht^{0.725} \times 71.84$).

Surface Area.—Max Rubner¹⁸ is the author of the law that states that the metabolism is proportional to the surface area of an animal. When the metabolism of various animals is calculated per unit weight, the variations are large. These variations, however, are minimized when metabolism is referred to surface area. For example, a horse weighing 441 kilos, produces 11.3 calories per kilo and 948 calories per square meter of the body surface; while a mouse weighing 0.08 kilo, produces 212 calories per kilo but only 1188 calories per square meter. Meeh¹⁹ was among the first to attempt to calculate surface area. The formula of Meeh was improved by DuBois and DuBois²⁰ who measured accurately the surface area of normal men and found that this can be expressed in terms of height and weight. Aub and

DuBois²¹ calculated the metabolism of normal subjects on such a basis and thus established standards of normal metabolism which are widely used.

CALORIES PER SQUARE METER OF BODY SURFACE PER HOUR (HEIGHT—WEIGHT FORMULA)

| Age, years. | Males. | Females. |
|---------------|--------|----------|
| 18 to 20..... | 41.0 | 38.0 |
| 20 to 30..... | 39.5 | 37.0 |
| 30 to 40..... | 39.5 | 36.5 |
| 40 to 50..... | 38.5 | 36.0 |
| 50 to 60..... | 37.5 | 35.0 |
| 60 to 70..... | 36.5 | 34.0 |

However, deviations from the Aub-DuBois standards have been noted especially in subjects representing extremes of height and weight. Because of these discrepancies, Harris and Benedict²² preferred to determine metabolism not on the basis of surface area, but rather on active protoplasmic mass. It is difficult to measure protoplasmic mass, although surface area may be an index of it. Omitting further theoretical considerations, it may be said that Harris and Benedict have developed a series of standards from a statistical treatment of height and weight data, which yields results that are largely in agreement with those obtained by Aub and DuBois. The Harris-Benedict standards are probably 2 to 4 per cent too high for modern methods, with trained subjects; those of Aub and DuBois may be 5 to 8 per cent too high.

An important addition has recently been made to this subject by Bohnenkamp and his coworkers.²³ They demonstrated that the heat lost by the body is not a direct function of the surface area, but rather of the effective radiating area; this may be measured by the sum of the surface areas of the body projected in the three planes, right to left, front to back and vertically; *i. e.*, the projections of a subject standing upright upon two adjacent walls, the floor and the ceiling. The sum of the projected areas is usually smaller than the actual surface area; and this is in accordance with the fact, for example, that the medial side of the extremities radiates heat back to the body and therefore diminishes the loss of heat. By this method, it may be possible to obtain values for metabolism which are not vitiated by the physical or pathologic peculiarities of the subject. Until more work is done with these new methods, which are still in the stage of development, it will be more convenient to use the standards of Aub-DuBois²¹ for the calculation of metabolism.

Basal metabolism is the expression of the combined energy required by all the cells of the body for maintaining the processes fundamental for life. The intensity of this requirement, however, depends upon the effective radiating area. A large body has a surface area which is smaller in proportion to its volume than has a small body; *i. e.*, the smaller the body, the greater its relative surface area. If the oxidations of the cells of a body are proportional to its surface area, then the cells of larger bodies should have a comparatively low rate of metabolism. It is, therefore, of interest that a given weight of muscle tissue excised from a rat has a smaller oxygen consumption than has the comparable tissue of a mouse.^{24, 25}

Basal Metabolism.—The metabolism of the body is subject to wide variations; for example, exercise may easily increase it several hundred per cent for short periods. Metabolism is also raised during digestion and in the effort necessary to maintain the temperature of the body in a cold environment. It is, therefore, most important to specify the conditions under which the observations are made. For this reason, conditions have been chosen for the determination of basal metabolism in which the energetic requirements are the minimal compatible with wakefulness. The subject is usually examined in the morning, fifteen to eighteen hours after eating; *i. e.*, in the postabsorptive state. He must first lie down quietly for thirty minutes in an equable environment to avoid the increase of metabolism due to exercise and the maintenance of the temperature of the body. These conditions are necessary not only for the determination of the basal oxygen consumption, but also for the basal carbon dioxide production; since in this way the influences which may cause either the retention of carbon dioxide in the body, or the expulsion of preformed carbon dioxide from bicarbonate, are avoided. The metabolism thus determined divided by the surface area of the subject is called the *basal metabolism*. It represents the energy required for the essential functions of life; *i. e.*, the energy for the muscles of respiration, the contraction of the heart necessary for circulation of the blood, the conduction of the nerve impulse and the secretion of glands.

In order to illustrate the principles involved in the determination of basal metabolism an actual calculation is presented:

Male subject, twenty-five years of age, 5 feet, 8 inches tall, weighing 150 pounds. During the observation, 0.6 Gm. of urinary nitrogen was excreted per hour and the gaseous exchange per hour was for carbon dioxide 13.50 liters and for oxygen 15.00 liters.

From the previous discussion of the urinary nitrogen (p. 477), it can be seen that the excretion of 0.6 Gm. of nitrogen in the urine is caused by the oxidation of an amount of protein (3.75 Gm.) which yields 15.9 calories, and a gaseous exchange of 3.55 liters of oxygen and 2.86 liters of carbon dioxide.

| | Carbon dioxide. | Oxygen. |
|---|-----------------|---------|
| Total gaseous exchange..... | 13.50 | 15.00 |
| Protein gaseous exchange..... | 2.86 | 3.55 |
| Nonprotein gaseous exchange..... | 10.64 | 11.45 |
| Nonprotein R. Q. of $\frac{10.64}{11.45}$ | = 0.93 | |

From table on page 478 1 liter of oxygen is equivalent to 4.961 calories at an *R. Q.* of 0.93. Therefore, 11.45 liters of oxygen is equivalent to 56.8 calories, of which 77.4 per cent are due to the oxidation of carbohydrate and 22.6 per cent to that of fat. The total metabolism will therefore be 15.9 calories (protein) + 56.8 calories (nonprotein) = 72.7 calories. The surface area obtained from the chart on page 479 is 1.8 square meters. Hence the basal metabolism is $\frac{72.7}{1.8} = 40.4$ calories per hour.

From the Aub-DuBois standards²¹ it may be seen that the usual value is 39.5 calorie per square meter per hour. The subject was therefore 0.9 calorie above the standard ($\frac{0.9}{39.5}$ or + 2 per cent).

The error of the method in a single determination may be as high as ± 15 per cent. However, the average of three successive observations or the average of the two of the three in best agreement is accurate to ± 10 per cent; and with a greater number of observations, the accuracy increases still further.

There are other methods for the determination of the metabolism. In one of these, the basal metabolism is determined by measurement of oxygen consumption alone. This may be done because protein metabolism is usually only 15 per cent of the total, and in the combustion of protein a liter of oxygen produces approximately the same number of calories per day in a man with a surface area of 1.5 square meters, a postabsorptive *R. Q.* of 0.82 is assumed. Furthermore, the caloric values of a liter of oxygen do not vary more than 7 per cent between 0.7 and 1. Returning to the above example, the oxygen consumption of 15 liters is, therefore, multiplied by 4.285; the caloric value of a liter of oxygen at the *R. Q.* of 0.82. This yields a total metabolism of 72.4 calories. Dividing by 1.8 square meters, the basal metabolism is 40.2, a value substantially the same as that obtained above after taking all the factors into consideration.

Another method for the determination of the metabolism depends upon the loss of weight due to "insensible perspiration" and the gaseous exchange. The "insensible perspiration" is the amount of water vapor (not frank perspiration) which diffuses through the skin.

Insensible loss of weight = H_2O (insensible perspiration) + $CO_2 - O_2$. DuBois²⁶ has found that under basal conditions in the calorimeter, 24 per cent of the heat lost by the body is the result of the evaporation of the insensible perspiration from skin and lungs. The extent of the gaseous exchange also varies with metabolism. Thus

all three variables are functions of metabolism. Accordingly, Benedict and Root²⁷ have been able to correlate the insensible loss of weight of a subject placed on a sensitive balance with the metabolic rate and have constructed prediction tables so that the metabolic rate may be estimated from the insensible loss of weight.

Factors Influencing Basal Metabolism.—*Physiologic Age.*—A basal metabolism of 40 calories per square meter per hour amounts to 1440 calories per day in a man with a surface area of 1.5 square meters. Usually, surface area is no greater than two square meters, and hence the basal rate for the day is less than 1900 calories. During the major part of adult life, basal metabolism is fairly constant; although there are, of course, both physiologic and pathologic variations. Age is one

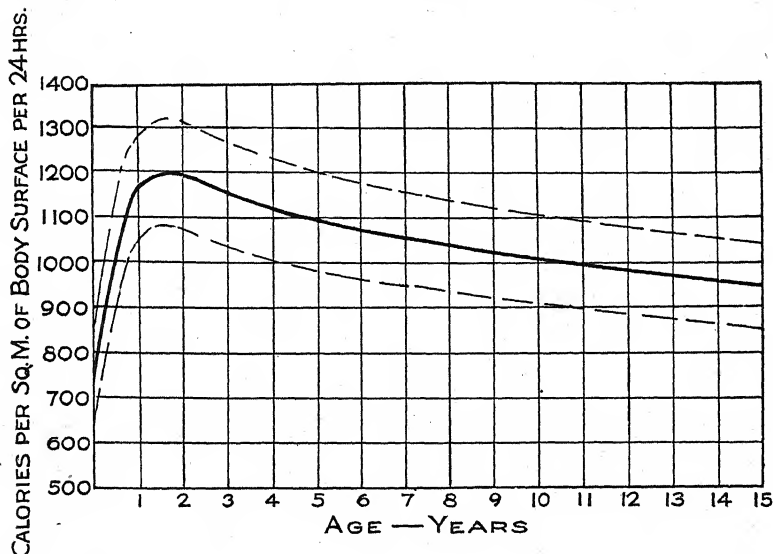


Fig. 33.—Basal metabolism of boys referred to age. (Talbot.³³)

of the most important factors; and a discussion of its effect may well begin with the influence of the fetus on the maternal metabolism.

A rise in metabolism has been found during pregnancy. One group of workers concluded that the increase in heat production may be accounted for by the added metabolism of the fetus, placenta and accessory structures, without a change in the intensity of heat production per unit mass of maternal tissue.²⁸ This point of view has been doubted by other observers who believe that the increased metabolism is not due simply to the summation of the effects of mother and fetus, but rather that the excess heat production during the latter part of pregnancy is a result of unknown mechanisms engendered by the state of pregnancy and involving other factors than those of fetal growth alone.²⁹

The metabolism of prematurely born infants is very low, approximately 25 calories per square meter per hour.^{30, 31} Infants born at term, however, possess a higher metabolism which approaches the value of 30 calories per square meter per hour.³² After the second day, the metabolism falls steadily for six days, when it begins to rise again. The fall occurs probably while the organism is adapting itself to extra-uterine life. The rise in metabolism thereafter is rapid and continues during the first and second years, attaining a value of 50 calories.³³ After this maximum there is a progressive decrease to 42 calories at the age of ten. During the prepubescent period, a second rise commences which finally reaches approximately +14 per cent, or 48 calories at its maximum about the time when the menses are established in girls and sexual maturity in boys.³⁴ This rise is followed by

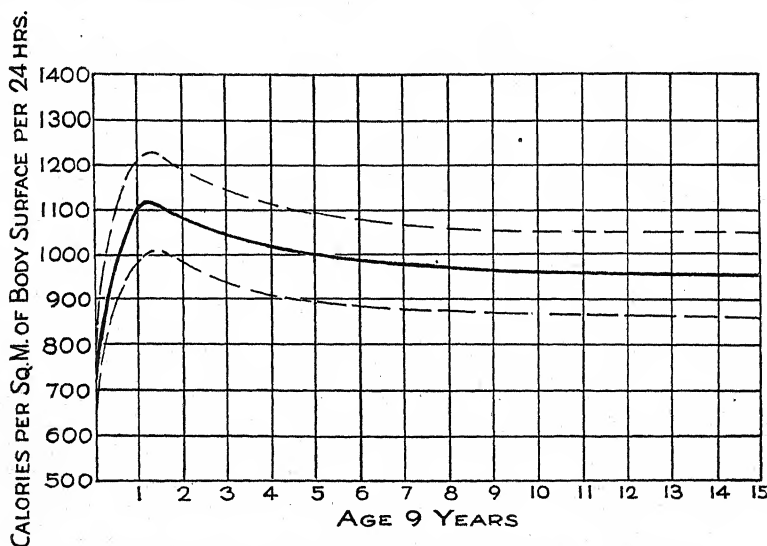


Fig. 34.—Basal metabolism of girls referred to age. (Talbot.³³)

a rapid postpubescent fall approximating values found before puberty. Then metabolism declines more slowly until it reaches a rate of 39.5 calories per square meter at the age of twenty. From the twentieth to the fortieth year metabolism does not change, and during the next three decades, the fall is not great; basal metabolism is respectively 38.5, 37.5 and 36.5 calories per square meter per hour.

Recent work indicates that after the age of seventy metabolism may decrease rapidly. Beyond the age of seventy-eight, a total metabolism of 1000 calories may be expected.³⁵ In one woman of one hundred and seven years, the calories per square meter of body surface per hour were as low as 21.25.³⁶

Sex.—Females exhibit a metabolism lower than males, a phenomenon which is definite even during the first year of life, long before

the maturation of the sex organs takes place. For example, at five years there may be a difference of approximately 100 calories per square meter per day. Since metabolism of the boys is 1100 calories per twenty-four hours, the girls are metabolizing at a rate which is 9 per cent lower than boys. The Aub-DuBois standards for women²¹ are, on the average, 7 per cent lower than those of men. It is quite likely that females require a lesser amount of oxidations to maintain their body temperature than do males. Sixty-five to 80 per cent of the heat loss of the body takes place by radiation and conduction both in man and in woman. The layer of subcutaneous fat in women, however, must be effective in diminishing their heat loss.

Physical Condition.—Lusk and DuBois³⁷ have demonstrated that "cage life" in dogs causes a marked reduction in basal metabolism because of the low state to which bodily functions may be reduced under such conditions. However, there is no demonstrable difference between reasonably normal individuals and subjects in athletic training. It should be remembered, however, that the total metabolism of the athlete is much higher, since the food intake must vary directly with the physical exertion of the individual.

Dietary.—The body appears able to adapt itself to straitened circumstances, since on a dietary restricted to 1950 calories basal heat production may be lowered from 15 to 20 per cent in a few weeks.³⁸ The character of the diet is also of importance. Vegetarians of six to eight years' standing possess a metabolism averaging 11 per cent below the Aub-DuBois standards.²¹ This change may be associated with the persistent decrease in the supply of amino acids and indicates an increased mechanical efficiency of the body.³⁹

Temperature.—Azorio de Almeida⁴⁰ observed a lower metabolism in tropical Brazil than obtains in the temperate zone. He believes that all factors which modify total metabolism will finally affect basal metabolism. The smaller difference between the temperature of the air and body, therefore, makes for a lower basal metabolism. Moreover, muscular work (and consequently food intake) is also diminished in the tropics.

Race.—The accepted standards of metabolism have been established from observations made on white North Americans. Colored Americans exhibit no deviations from these standards. On the other hand, female Tamils studied in India were found to have a metabolism on the average 17.4 per cent below the American standards. Malayales showed an average deviation of —16.1 per cent. Male and female Australian aborigines possess a basal metabolism of —14 per cent and —16 per cent, respectively. The metabolic rate of the Chinese is also less than that presented in the Aub-DuBois standards. Such decreases in metabolism are probably due to racial differences; for pure-blooded Chinese girls born in America and subsisting on American dietaries have a basal metabolism averaging —9 per cent.⁴¹

Aboriginal Americans exhibit the reverse deviation. Male Maya

Indians living in Yucatan have a basal of $+6.5$ per cent, and the male and female Mapuches of southern Chile, $+9.8$ and $+14.8$, respectively.⁴²

Sleep.—Sleep lowers the basal metabolism of North Americans by about 10 per cent, since with sleep comes a diminished oxygen requirement of most organs of the body.

Pathology.—Exophthalmic Goiter or Hyperthyroidism.—This is a constitutional disease finding somatic expression in an excessive secretion of a diffusely hypertrophied thyroid gland which causes, among other symptoms, an increased metabolism. A relationship exists between the height of metabolism and the severity of the disease; and in severe cases metabolism may be increased as much as 100 per cent.

Myxedema or Hypothyroidism.—When the secretion of the thyroid gland (*thyroxine*) is diminished by atrophy or operative interference, myxedema supervenes. This condition is characterized, among other symptoms, by a lowered basal metabolism. In the absence of the thyroid gland, the basal rate may be -40 per cent. If the thyroid gland fails to develop during fetal life, physical and mental development are stunted and basal metabolism is diminished. This condition is called cretinism. Plummer and Boothby⁴³ have shown that the intravenous injection of one milligram of crystalline thyroxine in patients with hypothyroidism causes an average increase of 2.8 per cent in the basal metabolic rate. The effect of such an injection is less in normal individuals, and may be negligible during hyperthyroidism.

Pituitary Gland.—Hyperfunction of the acidophile cells of the anterior lobe of the pituitary gland, causing gigantism before puberty and acromegaly in the mature patient, may produce a secondary hyperthyroidism and therefore an increased metabolism. Hypofunction of the diencephalic and pituitary mechanisms on the other hand may cause a diminished metabolic rate. In such conditions pituitary adiposity may develop.

With destructive lesions of the pituitary gland and the diencephalon, occasionally a condition of extreme emaciation accompanied by a diminished basal metabolism develops. At the present time, the effects of many newly discovered hormones such as those of the anterior pituitary lobe,⁴⁴ posterior pituitary lobe⁴⁵ and amniotic fluid⁴⁶ on metabolism are under active investigation.

Gonads.—The sexual difference in metabolism has been discussed above. There is no good evidence of diminution of metabolism as a result of castration. However, during menstruation uncomplicated by pain or distress, the metabolic rate may be decreased.

Adrenals.—A low metabolic rate is a frequent accompaniment of disease of the cortex of the adrenal gland.

Blood Diseases.—During leukemia and polycythemia, diseases characterized by the overproduction of white and red blood cells, respectively, basal metabolism may be as high as in severe hyperthyroidism.

Fever.—Van't Hoff found that with a rise of temperature of 10° C.

the velocity of chemical reactions increases 100 to 200 per cent. Metabolism should, if similarly governed, increase from 30 to 60 per cent for the three degrees between 37° and 40° C. DuBois⁴⁷ studied a large variety of fevers, pulmonary tuberculosis, erysipelas, arthritis, typhoid, malaria, and also the fever produced by intravenous injection of proteins foreign to the body, and found that the van't Hoff law applied to metabolism, since it increased about 13 per cent for each degree centigrade.

Diet.—In the computation of diets, basal metabolism must be taken into consideration since the basal requirements of most individuals constitute approximately half of the total caloric intake. The amount of physical exercise is also of importance. In sedentary life 2500 calories per day are sufficient to satisfy the needs of the body; 3000 calories are required for moderate physical effort. Farmers consume 3500 calories per day; and more food is necessary with occupations entailing greater physical exertion. Digestion and sleep also affect the caloric requirement. The extra heat expended during digestion, *i. e.*, the specific dynamic action of foodstuffs, is about 6 per cent of the basal rate. Sleep, on the contrary, depresses metabolism and does so by 10 per cent for approximately eight hours. Thus, 3000 calories may be taken as the average total requirement of the healthy individual.

| |
|---------------------------------|
| 1500 cal. basal metabolism |
| 1500 cal. exercise |
| 90 cal. specific dynamic action |
| —50 cal. sleep |
| 3040 cal. total metabolism |

The total requirement and not merely the basal requirement is the consideration in formulating dietaries.

During disease the requirements may be changed. Coleman and DuBois⁴⁸ showed that metabolism was raised from 36 to 40 per cent during the height of typhoid fever. However, in order to bring the patient into nitrogen equilibrium, the diet must exceed the theoretical requirement by 50 to 110 per cent chiefly in the form of nonnitrogenous foods. In prolonged fevers of whatever origin (in tuberculosis, for example) it is important to include the increased metabolism in the calculation of the dietary.

Adiposity, in most instances, is not the result of endocrine disturbance. It is due simply to lack of balance between intake and outgo, more food is ingested than is necessary for the maintenance of the individual. For the adiposity caused by excessive eating, the cure is obvious. Similarly, people who are underweight because of functional causes may be improved by increasing the caloric intake. The ingestion of increased amounts of vitamin B and the injection of insulin may aid in increasing weight.⁴⁹ However, when gross abnormalities of weight are due to endocrine or other organic changes, the conditions cannot be remedied merely by variations of the caloric intake; the

etiologic factor must also be treated. Nevertheless, the diet should be appropriate. For example, the caloric intake must be greatly increased during exophthalmic goiter.

Summary.—The subject of basal metabolism has been briefly reviewed; its cause, the fundamental processes involved, and the principles of the methods of determination. Basal metabolism is defined as the minimal amount of energy required to maintain life when the subject is awake, postabsorptive, and at perfect rest in an equable temperature. In the computation of diets, it is necessary to include normal variations due to age, sex, race, and previous dietary, as well as the pathologic deviations occurring during fevers (certain diseases of the blood, and also disturbances of endocrine origin, *i. e.*, thyroid, pituitary and adrenal glands). Despite the numerous factors which affect basal metabolism, its most important characteristic is its constancy. This makes any deviation from the standard values all the more significant.

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REFERENCES

1. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*, Vol. 1, (1931).
2. Peabody, F. W., and Wentworth, J. A.: *Arch. Internal Med.*, 20, 443 (1917).
3. Hamilton, W. F., and Morgan, A. B.: *Am. J. Physiol.*, 99, 526 (1932).
4. Adrian, E. D.: *J. Physiol.*, 79, 332 (1933).
5. Heymans, C., and Bouckaert, J. F.: *J. Physiol.*, 69, 254 (1930).
6. Schmidt, C. F.: *Am. J. Physiol.*, 102, 119 (1932).
7. Heymans, J. F., and Heymans, C.: *Arch. Int. Pharmacol. Therap.*, 33, 273 (1927).
8. Selladurai, S., and Wright, S.: *Quar. J. Exper. Physiol.*, 22, 285 (1932).
9. Gemmill, C. L., and Reeves, D. O.: *Am. J. Physiol.*, 105, 487 (1933).
10. Anson, M. L., and Mirsky, A. E.: *Physiol. Rev.*, 10, 506 (1930).
11. Keilin, D.: *Proc. Roy. Soc. (London)*, 104B, 206 (1929).
12. Warburg, O., and Megelein, E.: *Z. Electrochem.*, 35, 928 (1924).
13. Wieland, H.: *Ber.*, 46, 3327 (1913).
14. Meldrum, N. U., and Roughton, F. J. W.: *J. Physiol.*, 80, 113 (1933).
15. Lusk, G.: *Science of Nutrition* (1928).
16. Boothby, W. M., and Sandiford, I.: *Laboratory Manual of the Technic of Basal Metabolic Rate Determinations* (1920).
17. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*, Vol. 2 (1932).
18. Rubner, M.: *Energiegesetze* (1902).
19. Meeh, K.: *Z. Biol.*, 15, 425 (1879).
20. DuBois, D., and DuBois, E. F.: *Arch. Internal Med.*, 17, 863 (1916).
21. Aub, J. C., and DuBois, E. F.: *Arch. Internal Med.*, 19, 823, 840 (1917).
22. Harris, J. A., and Benedict, F. G.: *Carnegie Inst. Washington. Pub. No. 279* (1919).
23. Bohnenkamp, H., Ernst, H. W., Pasquay, W., and Schwab, J.: *Arch. ges. Physiol.*, 238, 40, 63, 79, 100, 125 (1931).
24. Meyerhof, O., and Himwich, H. E.: *Arch. ges. Physiol.*, 205, 415 (1924).
25. Wels, P.: *Arch. ges. Physiol.*, 209, 32 (1925).
26. Gephart, F. C., and DuBois, E. F.: *Arch. Internal Med.*, 17, 902 (1916).
27. Benedict, F. G., and Root, H. F.: *Arch. Internal Med.*, 38, 1 (1926).
28. Sandiford, I., Wheeler, T., and Boothby, W. M.: *Am. J. Physiol.*, 96, 191 (1931).
29. Rowe, A. W., and Boyd, W. C.: *J. Nutrit.*, 5, 551 (1932).
30. Marsh, M. E., and Murlin, J. R.: *Am. J. Diseases Children*, 30, 310 (1925).
31. Talbot, F. B., Sisson, W. R., Moriarty, M. E., and Dalrymple, A. J.: *Am. J. Diseases Children*, 24, 95 (1922).

32. Murlin, J. R., Conklin, R. E., and Marsh, M. C.: *Am. J. Diseases Children* 29, 1 (1925).
33. Talbot, F. B.: *Am. J. Diseases Children*, 21, 519 (1921).
34. Topper, A., and Mulier, H.: *Am. J. Diseases Children*, 43, 327 (1932).
35. Benedict, F. G., and Meyer, M. H.: *Proc. Am. Phil. Soc.*, 11, 143 (1932).
36. Hitchcock, F. A., and Matson, J. R.: *Am. J. Physiol.*, 105, 52 (1933).
37. Lusk, G., and DuBois, E. F.: *J. Physiol.*, 59, 213 (1925).
38. Benedict, F. G., Miles, W. R., and Smith, H. M.: *Carnegie Inst. Washington*. Pub. No. 280 (1919).
39. Wakeham, G., and Hansen, L. O.: *J. Biol. Chem.*, 97, 155 (1932).
40. Azorio de Almeida, A. O.: *J. Physiol. Path. Gen.*, 22, 1248 (1924).
41. Benedict, F. G.: *Am. J. Physical Anthropol.*, 16, 463 (1932).
42. Pi-Suner, J.: *Am. J. Physiol.*, 105, 383 (1933).
43. Boothby, W. M.: *Oxford Medicine*, 3, 948 (1923).
44. Foster, G. L., and Smith, P. E.: *J. Am. Med. Assocn.*, 87, 215 (1926).
45. Himwich, H. E., and Haynes, F. W.: *Am. J. Physiol.*, 96, 640 (1931).
46. Sherwood, T. C., Savage, M., and Hall, J. F.: *Am. J. Physiol.*, 105, 241 (1933).
47. DuBois, E. F.: *Basal Metabolism in Health and Disease* (1927).
48. Coleman, W., and DuBois, E. F.: *Arch. Internal Med.*, 15, 887 (1915).
49. Nahum, L. H., and Himwich, H. E.: *Am. J. Med. Sci.*, 183, 608 (1932).

CHAPTER XVIII

ANIMAL PIGMENTS

AMONG the various pigments of the animal organism, the colors displayed by three have come to be used in common speech to express similes. We say "red as blood," "green as bile" and "black as a negro." These phrases we learned long before the actual chemical pigment substances present in blood, bile or skin had been isolated. They will serve here to sum up for us the three chief pigment types of the animal organism: The *hemoglobin* group, the chemically allied *bile* group, and the *melanin* group. Besides the chief loci, the blood, bile and epidermal tissues, we shall find pigments in the urine, muscles, body lipoids, iris, retina and indeed in all the cells of the body (cytopigments or cytochromes, lipochromes and lyochromes). A group of other pigments are to be found in the lower animals, but some of the chief pigment types of the primates are to be found widely distributed in nature, not only in lower animals but also in plants.

PYRROLE PIGMENTS

Heme Pigments and Porphin Pigments.—If we consider the first great group of the pyrrole pigments, we find that most of the blood, urine and bile pigments, together with a type of cytopigment, the cytochromes, are all related; they are pyrrole pigments of one sort or another. The pyrrole pigments in which the common structure is that of a porphin, and more especially that of a porphyrin (defined below), constitute the major group of these pyrrole dyes of the organism. The bile pigments, while constituted of pyrrole nuclei, are not of the porphin series.

Indeed, the porphyrin family have another great branch; for one of the porphyrins, aetioporphyrin, has been found to be the connecting link between the blood (hemin) pigments and the chlorophyll pigments of the plant world. It has long been known that chlorophyll and hemoglobin are both pyrrole dyes, the one a complex with magnesium and phytol, the other with iron and globin; but it was only with the discovery of the identity of this "aetioporphyrin," which could be obtained from either hemin or chlorophyll, that the very considerable body of work which had been assembled in the study of chlorophyll could be made use of for the study of the blood porphyrins and hemins. With the final building up of a careful groundwork by the synthesis and study of the chemical properties of pyrrole bases and carboxylic acids by Hans Fischer, and his development of methods of synthesis of these into pyrrolemethenes and porphyrins,

it became possible to effect the proof of the structure of hemin. The exact nature of the linkage of the iron in the porphyrin to make the hemin is still in some doubt; for it appears to be concerned with a complex formation involving secondary valences, *i. e.*, a molecular compound. However, the structure of the carbon heterocycle to which the iron is attached is now well established.

The history of advance in this field has been made chiefly by Hoppe-Seyler, Nencki, Küster, Piloty, Willstätter, and Hans Fischer. The theories of structure have been many and confusing; and it seems best today to set them aside, and set forth the structure and proof of structure without concern for the older views. The chief contributions of the above-named men may be briefly summarized as follows: Hoppe-Seyler prepared porphins from chlorophyll (1879-1881); Küster studied the oxidative degradation of hemin; Nencki and Piloty, the reduction products; Willstätter studied porphyrin chemistry chiefly from the chlorophyll viewpoint; Fischer studied the products of exhaustive methylation, made (by synthesis) the pyrrole building stones and finally synthesized porphyrins and hemes.

Hemoglobin.—The best known among the pyrrole pigments of the animal is the protein *hemoglobin*, contained in the red blood cells of those animals possessing them. Hemoglobin consists of a protein portion and a dyestuff portion, or "prosthetic group." Hemoglobin was the first protein to be crystallized. Indeed, it was not necessary artificially to produce these crystals, for the shining crystals of this protein could be seen, for example, in the blood puddings or blood sausages of the European peasant, long before a physiology or chemistry of the blood existed.

Hemoglobin possesses the power of combining with oxygen to form oxyhemoglobin and to yield up this oxygen wherever there is a decrease of oxygen in the solvent. The change in the visible absorption spectrum which occurs shows that a definite compound is formed. This change can be seen by the eye in the color change from dark purple to bright red. One gram of hemoglobin crystals can combine with 1.34 cc. oxygen at 0° and 760 mm. It is, of course, this oxygen-carrier property of hemoglobin, rather than its dyestuff properties, or indeed its iron catalyst properties, which lend the compound its enormous physiologic significance. Indeed, it might be noted that the light-absorbing properties of most of the heme pigments, as far as we know today, seem to be without much functional importance to the organism. One must admit, however, that the unsaturated structural configurations which result in color (*i. e.*, in visible light absorption), possess properties as carriers or catalysts which are of importance for their biological function.

The first question of the chemist concerning a chemical is its percentage composition and then its degradation fragments. Hemoglobin analyses are usually reported on the oxy-form. For different animal species the values are slightly different, but the figures of Abderhalden

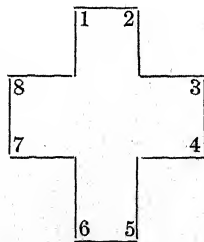
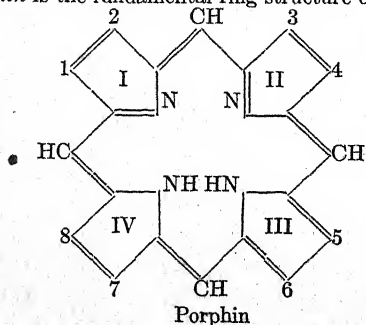
for horse oxyhemoglobin may be cited: Carbon 54.75, hydrogen 6.98, nitrogen 17.35, sulfur 0.42, iron 0.38, oxygen 20.12. A typical empirical formula is $C_{712}H_{1130}N_{214}S_2FeO_{245}$ (Zinoffsky). Osborne in 1902 reported the minimum possible molecular weight to be 16,721–16,655. Svedberg, by his ultra centrifuge method, gave in 1927 the value 68,000, as have others by other modern methods. Four hemochromogen molecules of molecular weight 17,000 are probably associated in the hemoglobin molecule.

On acid or alkaline hydrolysis, hemoglobin breaks down into the protein *globin* (94 per cent) and the iron pigment or prosthetic group, *protohematin* (6 per cent). Some call the latter *heme*.^{*} The protein, globin, is usually regarded as a histone.[†] The linkage to globin is known not to be through the unsaturated side-chain of the heme. Just where it is attached is not established. The *protohematin*, $C_{34}H_{32}O_4N_4FeOH$, on strong hydrolysis breaks down into an iron-free pigment, *hematoporphyrin*, $C_{34}H_{38}O_6N_4$, and then into a series of porphyrins leading to an *aetioporphyrin*, $C_{32}H_{38}N_4$.

Methemoglobin is very readily formed from hemoglobin by the action of mild oxidizing agents. It is an oxidation product. Küster and Conant have assumed that in this substance the iron is oxidized so that the iron carries an OH group. Some write this substance in the form HbO ; others, $HbOH$. It is readily distinguished from oxy- or reduced hemoglobin by its different absorption bands.

No attempt has been made to describe the spectrographic properties of the heme pigments, for they are so frequently and thoroughly discussed and can be found in many standard works and tables of constants. Before the elucidation of the chemical structure of these compounds, the spectrographs were often the only means of distinguishing the pigments.

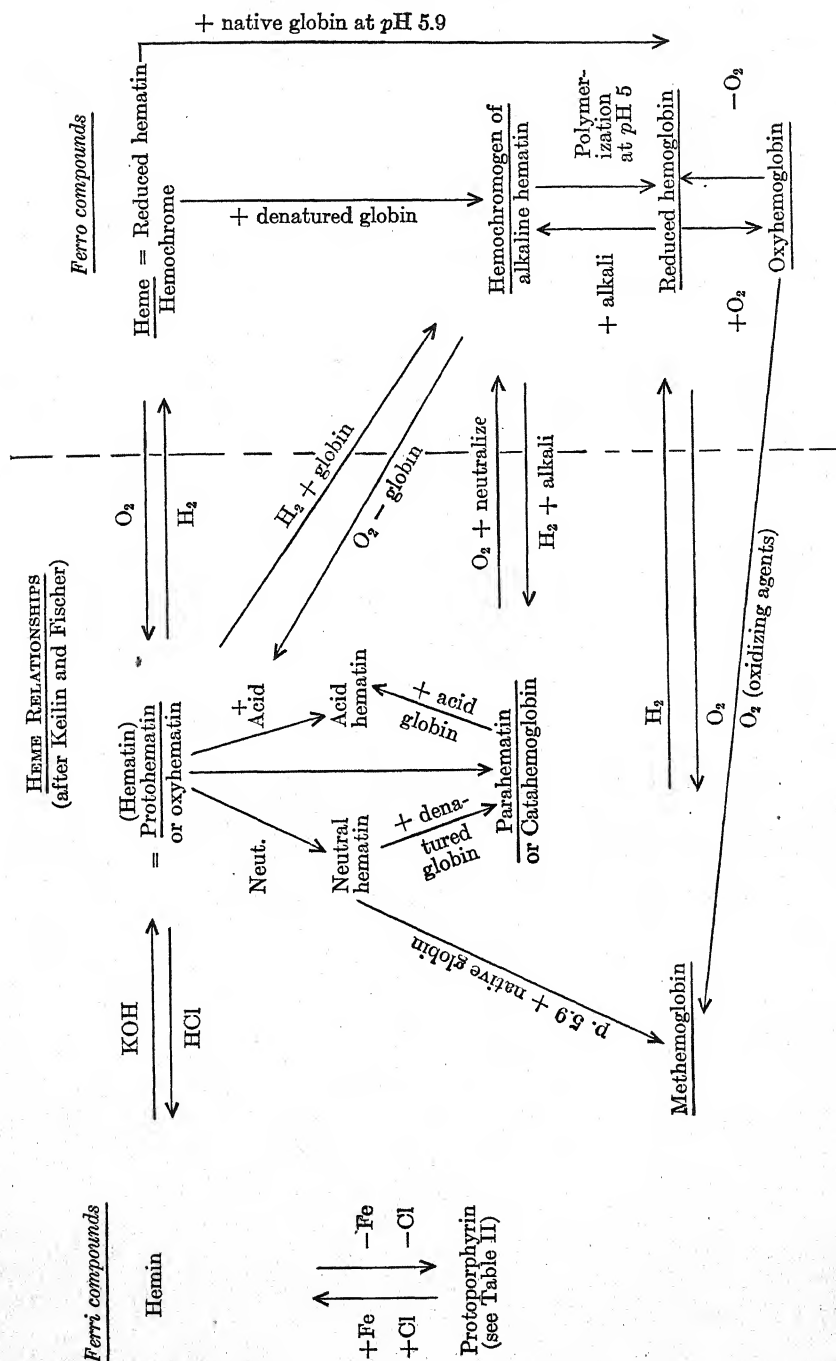
^{*} Nomenclature: *Heme* is reduced hematin, also called hemochrome. Hemes as a class are iron porphyrins. *Porphyrins* are substituted porphins free from iron. *Porphin* is the fundamental ring structure of four linked pyrrole nuclei.



(Porphin in a chemical shorthand devised by Fischer.)

When porphins are oxidized they yield derivatives of rhodins and *chlorins* which are also formed by degradation of chlorophylls. The latter appear to be magnesium complexes of derivatives of chlorins linked to the alcohol, phytol.

[†] It contains histidine as the principal diamino acid, whereas in other histones, the principal diamino acid is arginine.

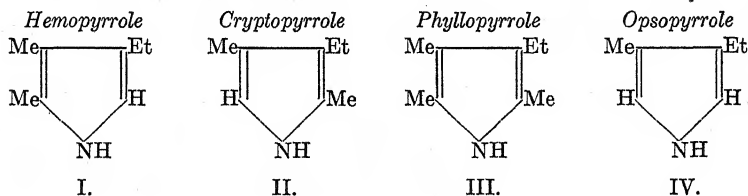


Protohematin hydrochloride is known as *hemin*. Protohematin can be formed by the peptic digestion of hemoglobin. It has been found free in animal muscle and also combined in the compound cytochrome. *Hemin*, $C_{34}H_{32}O_4N_4FeCl$, is formed by treating oxyhemoglobin with acetic acid and sodium chloride at $105^\circ C$. It has been obtained in crystalline form from yeast (Fischer) and has been spectroscopically identified in plants. It has been recognized in catalase preparations.

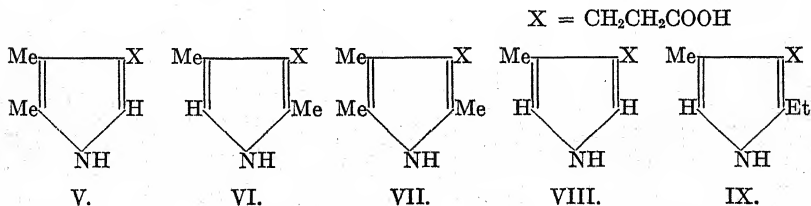
When hemin is reduced by hydrogen in the presence of colloidal palladium, it takes up four atoms and becomes *mesohemin*, $C_{34}H_{36}O_4N_4FeCl$; when reduced with hydrogen iodide and phosphorus iodide, iron splits off and *mesoporphyrinogen*, $C_{34}H_{42}O_4N_4$, a colorless compound, is formed. If hemin is treated with hydrogen bromide and glacial acetic acid, *hematoporphyrin*, $C_{34}H_{38}O_6N_4$, is formed. If the latter is reduced with concentrated methyl alcohol, potassium and pyridine at $20^\circ C$. it gives *hemoporphyrin*, $C_{34}H_{38}O_4N_4$. This treated with soda lime splits off CO_2 and becomes *aetioporphyrin*, $C_{32}H_{38}N_4$. Some of these relations are shown in the table on page 494.

The Degradation of the Hematin Nucleus to Pyrrole Fragments.—

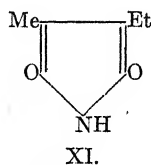
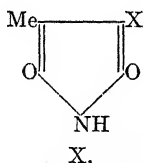
If hemin is drastically treated, various fragments are found in the resulting mixture. Küster studied the effect of oxidation, Nencki, that of reduction with hydriodic acid and acetic acid, and Fischer degradation by exhaustive alkylation. Thus, reduction with hydriodic acid and acetic acid yields a mixture of acids and bases. The following pyrrole bases are obtained:



Piloty investigated the acid fraction and found therein the corresponding pyrrolecarboxylic acids (Structures V–VIII), as well as xanthopyrrole carboxylic acid, (IX).

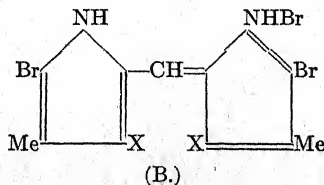
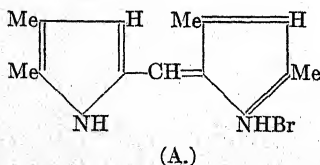


The constitution of all these building stones of hemin has been demonstrated by their synthesis. Knorr and Hess synthesized cryptopyrrole by reduction of 2,4-dimethyl-3-acetypyrrole. Fischer and Bartholomäus then synthesized phyllopyrrole and its carboxylic acid. Piloty and Blömer then made hemopyrrole. Synthesis of the other pyrrolecarboxylic acid and of opsopyrrole followed from Fischer's laboratory. Küster, by the oxidation of hemin, obtained hematic acid (X), and methylethylmaleinimide (XI).

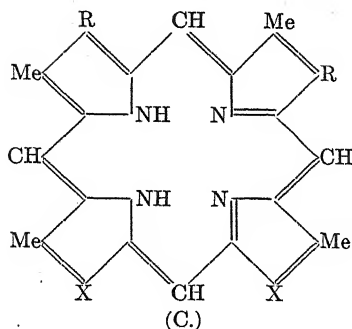


Fischer, by exhaustive methylation of hemin, obtained phyllopyrrole and its corresponding carboxylic acid. From all the above work it becomes apparent that four pyrrole nuclei must exist in hemin, two basic and two acidic rings. Direct molecular determinations on hemin showed the molecular weight to be about 600. Fischer now began the systematic synthesis of dipyrrolemethenes and soon found that by brominating the nucleus he could join the brompyrrolemethenes in porphyrins. Then it became a question which of many possible porphyrins would turn out to be the protoporphyrin that, with iron, would give hemin.

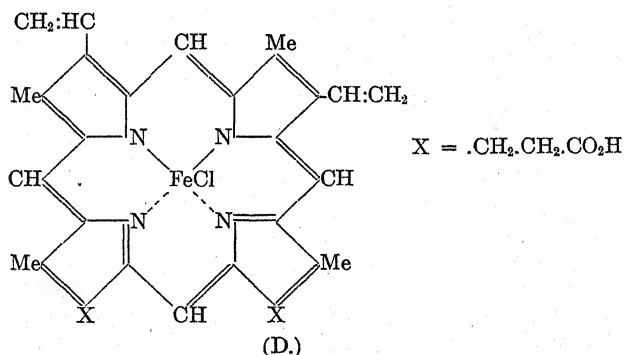
Synthesis of Hemin.—2,3-Dimethylpyrrole and 2,4-dimethylpyrrole-5-aldehyde were synthesized and condensed by means of alcoholic hydrobromic acid to 4,5,3',5'-tetramethylpyrrolemethene hydrobromide (A). Another methene was now made by bromination of cryptopyrrole carboxylic acid and condensation of two mols with loss of a methylene group, giving 5,5'-dibromo-3,3'-di- β -carboxyethyl-4,4'-dimethylpyrrole-2,2'-methene hydrobromide (B). In this and the following formulas X is the "propionic acid group" or β -carboxyethyl group, *i. e.*, $X = CH_2CH_2COOH$, unless otherwise indicated.



The two methenes, A and B, were next heated together in a succinic acid melt at 180° – 190° C. to form the porphyrin, *deuteroporphyrin* (C) (here $R = H$):



This porphyrin was now converted into deuterohemin by treating it with ferrous acetate, acetic acid, sodium chloride and hydrochloric acid. The introduction of the iron renders the pyrrole nucleus more reactive and now, on treatment with acetic anhydride in the presence of stannous chloride, diacetylduterohemin was formed (R becomes COCH_3). Reducing this with alcoholic potassium hydroxide, it lost iron and yielded the di-secondary alcohol, hematoporphyrin (R becomes CHOHCH_3). This proved to be identical with the hematoporphyrin obtained from natural hemin. It was next converted to protoporphyrin by heating in a high vacuum at 105°C . (R becomes CH:CH_2 , the vinyl group). With ferrous iron this gave hemin (D) identical with that from hemoglobin. The structure of hemin, as determined by this synthesis, is thus:



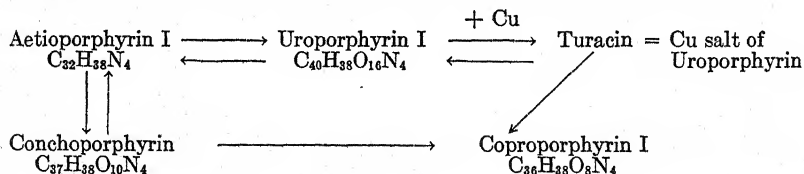
It will be seen that the four pyrrole nuclei are joined (at the α carbons) by methene groups. A system of conjugated double bonds accounts for the color. The iron is held by extra valences in a complex nonionic combination. One may now see what happened on strong oxidation. The nuclei bearing the vinyl group were completely decomposed, but the nuclei bearing the propionic group appeared as hematic acid.

By a similar synthesis, Fischer has proved the structure of the important natural and derived porphyrins. They are tabulated below ($X = \text{CH}_2\text{CH}_2\text{COOH}$):

| | Isomerides. | | Side chains. |
|--|------------------|---------------------|----------------------------|
| | Possible number. | Synthesized number. | |
| Hemin..... $\text{C}_{34}\text{H}_{32}\text{O}_4\text{N}_4\text{FeCl}$... | 15 | 2 | 4Me 2X 2CH:CH ₂ |
| Protoporphyrin..... $\text{C}_{34}\text{H}_{34}\text{O}_4\text{N}_4$ | 15 | 2 | 4Me 2X 2CH:CH ₂ |
| Hematoporphyrin... $\text{C}_{34}\text{H}_{38}\text{O}_6\text{N}_4$ | 15 | 2 | 4Me 2X 2CH(OH)Me |
| Mesoporphyrin..... $\text{C}_{34}\text{H}_{38}\text{O}_4\text{N}_4$ | 15 | 12 | 4Me 2X 2Et |
| Aetioporphyrin..... $\text{C}_{32}\text{H}_{38}\text{N}_4$ | 4 | 4 | 4Me ... 4Et |
| Deuteroporphyrin... $\text{C}_{30}\text{H}_{30}\text{O}_4\text{N}_4$ | 15 | 3 | 4 Me 2X ... |
| Deuterohemin..... $\text{C}_{30}\text{H}_{28}\text{O}_4\text{N}_4\text{FeCl}$.. | 15 | 2 | 4Me 2X ... |

There are four possible aetioporphyrins, all of which Fischer has synthesized. Only two are known to be obtainable from natural products. Aetioporphyrin III, or 1,3,5,8-tetramethyl-2,4,6,7-tetra-ethylporphin, is obtainable from hemin and chlorophyll, and is the basis of the above-tabulated porphyrins. Three porphyrins, derived from the isomeric aetioporphyrin I (1,3,5,7-tetramethyl-2,4,6,8-tetra-ethylporphin) are derived from natural sources.* From the four aetioporphyrins may be derived fifteen possible mesoporphyrins; and Fischer has synthesized at least twelve of these in the course of his work.

RELATION OF NATURALLY OCCURRING DERIVATIVES OF AETIOPORPHYRIN I



The *function of hemoglobin* in the blood is concerned with its oxygen-combining powers. Heme is so insoluble in water that it cannot

* In the strange and rare disease, porphyria, either congenital or resulting from sulfone toxicity (sulfonal), coproporphyrin I and uroporphyrin I appear in the excreta and coprobilirubin in the bile (see below). A coproporphyrin III has also been found in the urine and feces of a porphyria human. Coproporphyrin I is present in traces in the feces of normal individuals and has been found in yeast. Turacin, a pigment of bird's feathers, is a derivative of uroporphyrin I.

act as an efficient gas carrier; but the globin compound possesses the desirable properties. Just as there can be oxyhemoglobin, there can be CO-hemoglobin and HS-hemoglobin. Cyanides, however, first convert hemoglobin to methemoglobin, then combine with the latter to form cyanhemoglobin. Fluorine, similarly, first forms methemoglobin, then combines with it. The physical chemistry of the gaseous exchange of plasma and blood pigment has already been discussed (see previous chapters).

Cytochrome.*—A hematin compound exists in the cells of all aerobic organisms, both plants and animals, and is named *cytochrome*. McMunn found spectroscopically a hemochromogen-like substance in muscles and tissues of animals which he called myohemin or *histohemin*. Keilin rediscovered this and named it cytochrome. Since it is found widely distributed in all sorts of cells, this latter is a more generic name. It is a sort of universal cytorespiratory ferment. Keilin found that there exists in cells free protohematin, a hemochromogen and three hemochromogen-like substances which he called cytochrome a', b' and c'. These can exist as oxycytochromes and reduced cytochromes.

The reduced form of the mixture has four characteristic strong absorption bands at $604.5m\mu$ (a), 566.2 (b), 550 (c) and 520.4 (d). The oxycytochrome has weak bands at 566.6 and at 528.7 . Bands a, b, and c represent the *alpha* bands of the three components, while d is the fused *beta* band of b' and c'. Compound c' is extractable from muscle or yeast with water. Unlike hemochromogens, none of these can combine with carbon monoxide at physiologic pH's. A hematoporphrin derived from c' has been shown to be identical with protoporphyrin obtained from hematin. Little is yet known of the nitrogenous groups forming hemochromogens from heme in these cytochromes. Cytochrome is reduced in the living cell by various hydrogen donors. Cytochrome acts as a hydrogen acceptor which is specifically oxidized by indophenol oxidase. Other rôles (such as bio-oxidation carriers) are also postulated for the cell hematin and the cytochrome components.

Warburg's Respiratory Ferment.—Warburg has found in cells a respiratory ferment of universal distribution, which has an absorption band at a slightly different point from that of hemin, but which he believes to be an oxidized hemin complex. He notes various points differentiating it from cytochrome, in particular that it is sensitive to carbon monoxide while cytochrome is not. Its ferment powers are destroyed by high concentrations of carbon monoxide and by much smaller quantities of cyanide.† In yeast the concentration of the ferment is not over 4×10^{-7} Gm. per gram of yeast.

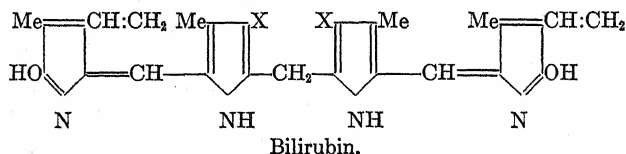
* See also Chapters XVII and XIX.

† The toxic effect of cyanide on higher organisms has to do with the poisoning of this ferment system of the cells. The toxic power of carbon monoxide, however, arises from the reaction of the monoxide with the blood iron.

Muscle Hemoglobin or Myochrome.—A hemoglobin differing slightly from blood hemoglobin is the red pigment to be found in the fibers of striated muscle. The spectral bands are shifted slightly toward the red. The protein fraction is considered to differ from blood globin, the prosthetic group to be the same.

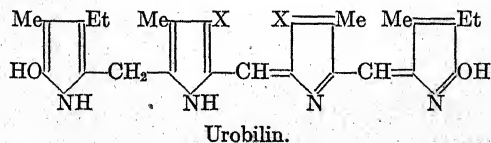
Bile and Urine Pigments.—*Bilirubin* is a bile pigment, $C_{33}H_{36}O_6N_4$, supposed to arise from the breakdown of hemoglobin in the cells of the reticulo-endothelial tissue, especially of the liver. It is found in oxgall. It has no iron and contains one less carbon atom but two more oxygen atoms than hemin. On energetic oxidation, it gives hematic acid and on reduction with hydrogen iodide it gives cryptopyrrole and cryptopyrrolecarboxylic acid (see above). It has been shown by Hans Fischer to be a compound composed of two pyrrole-methenes joined by a methylene group. It thus can be conceived to form by splitting open of the porphin ring of a meso- or protoporphin. The formula as deduced by Fischer from the degradation products is given below:

(X = CH_2CH_2COOH in this and the following formulas.)

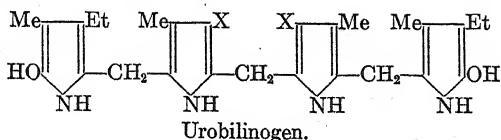


This unsymmetrical structure of bilirubin is that which one would expect to form from the mesoporphyrin of hemin. The physiological derivation of bilirubin from hemin can occur by oxidation of the α -methene linkage, *i. e.*, between the pyrrole nuclei I and II.

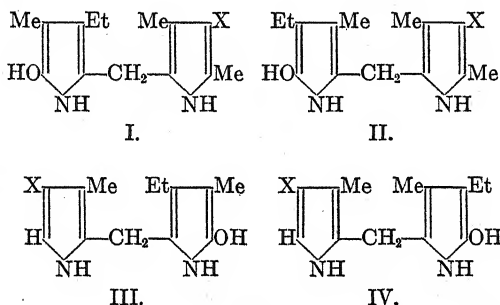
In the intestine, putrefactive bacteria convert (reduce) bilirubin to urobilirubin and urobilin (stercobilin). Catalytic reduction and sodium amalgam reduction turn bilirubin into the pyrrolemethene, meso-bilirubin, $C_{33}H_{40}O_6N_4$ (in the above formula the vinyl groups become ethyl groups) and dihydromesobilirubin, $C_{33}H_{42}O_6N_4$. Still stronger reduction produces a pyrrolemethane, mesobilirubinogen, which is another name for *urobilinogen* $C_{33}H_{44}O_6N_4$. From Fischer's work the most likely formula for urobilin is



and for urobilinogen is



On oxidation, mesobilirubin yields hematic acid and methyl-maleinimide just as does mesoporphyrin (as is to be expected of the open-chain homologue of the mesoporphyrin). On reduction with hydrogen iodide and acetic acid, it yields bilirubic acid (I), isobilirubic acid (II), neobilirubic acid (III) and isoneobilirubic acid (IV). III and IV lack the methyl group of I and II. The iso-forms have reversed methyl group and ethyl group positions on the pyrrole ring bearing both these groups.

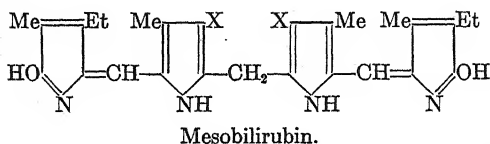


These acids on further reduction give cryptopyrrole. Their acidic reduction products are, however, different, the bilirubic acids giving cryptopyrrole carboxylic acid, while the neobilirubic acids give hemopyrrole carboxylic acid. They are the leukobases of four dyes, xanthobilirubic acid, isoxanthobilirubic acid, neoxanthobilirubic acid, and isoneoxanthobilirubic acid, into which they can be converted by treatment with alkali methoxides. Fischer has synthesized these.

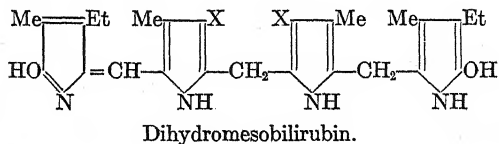
As an example, may be cited the synthesis of bilirubic acid, namely, half the bilirubin molecule. This was done in the following way: 5-Aldehyde-3-methyl-4-ethylpyrrole-1,2-carboxylic acid and cryptopyrrolecarboxylic acid condense in the presence of excess hydrogen bromide to give a 5-carboxy compound, which, on bromination, gives 5-bromo-4,3',5'-trimethyl-3-ethylpyrrole-methene-4'-propionic acid hydrobromide. By replacement of Br with OH, this is converted into xanthobilirubic acid and thence to bilirubic acid by bromination and treatment with pyridine.

Similarly, the others can be synthesized and each can be converted to a mesobilirubin. On condensation with formaldehyde, two mols of neoxanthobilirubic acid condense to give a mesobilirubin, but not the

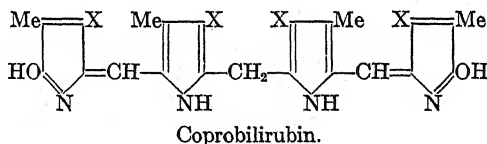
natural one. Fischer has shown the mesobilirubin of natural bilirubin to be



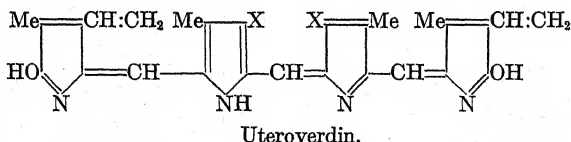
Dihydromesobilirubin is



If the vinyl groups of bilirubin are replaced by propionic groups a coprobilirubin is formed. Another coprobilirubin has been found to occur in certain pathologies and has been synthesized by Fischer.

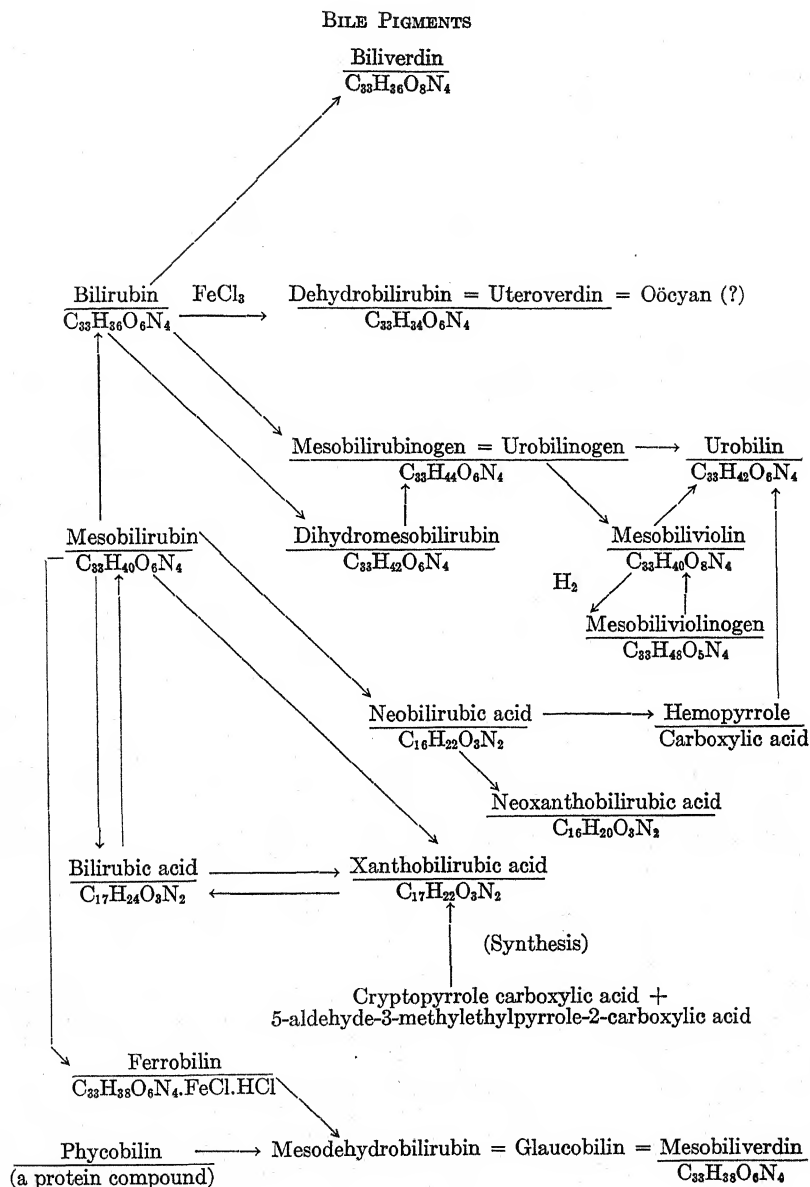


It may be noted that this coprobilirubin is the open-chain analogue of coproporphyrin I, in which the same sequence of four, propionic acid group substituted, pyrrole nuclei are joined in a ring. On oxidation of bilirubin with ferric chloride, dehydrobilirubin, also known as uteroverdin, $C_{33}H_{34}O_6N_4$, is formed. This is found in the dog placenta and as a pigment in bird's eggs, and is obtained as a by-product in the extraction of bilirubin from gallstones. Its formula has been suggested as

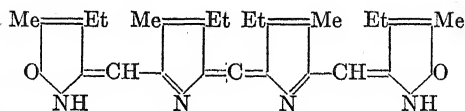


Biliverdin is an oxidation product of bilirubin, found in cadaver bile, meconium and in dog placenta. Its empirical formula is $C_{33}H_{36}O_8N_4$. A table of relations among the bile pigments is appended (p. 503).

The Nature of the Gmelin Reaction.—It is characteristic of the bile pigments to give the *Gmelin* reaction with concentrated, fuming



nitric acid. A color change occurs from blue to green, to violet, to red and to orange yellow; and finally there is decolorization. Fischer has studied this reaction and concludes that a dehydrogenation of a four-pyrrole nucleus occurs in successive steps, ending in a holoquinoid system, which in a given instance he formulates as



The above is the end-result of the Gmelin reaction oxidation of an aetiomesobilirubin which Fischer has synthesized and which is a decarboxylated mesobilirubin (*i. e.*, the propionic groups become (C_2H_5)).

Heme and Porphyrin Pigments of Lower Organisms.—**Hemocyanin**,* a blue dye, occurs in the blood of mollusca and arthropoda, and is an oxygen-carrier pigment of these lower forms. The dye has a molecular weight of 16,640 and is composed of a protein and a prosthetic group. The latter appears to be a porphyrin complex with copper (about 7 per cent Cu). The crystalline protein contains about 0.34–0.35 per cent copper, varying according to the species. This respiratory pigment has about one fourth the combining capacity for oxygen shown by hemoglobin.

Pinna globin is a brown oxygen-carrier pigment occurring in the blood of the lamellibranch, *Pinna squamosa*. The dye is very like hemocyanin in properties except that it contains manganese instead of copper. *Chlorocruorin* is a green oxygen-carrier pigment contained in the blood of polychaete worms. It has been obtained in crystalline form from this source. The prosthetic group is a porphyrin combined with iron but is not heme itself (Fox). *Hemoerythrin* is a red pigment occurring in the blood of worms. It does not yield hemin but is believed to be a porphyrin complex. In ascidians a brown, *vanadium-containing* oxygen-carrier pigment has been reported. *Helicorubrin* is a red pigment occurring in the liver and gut of molluscs and in the crayfish. It is a naturally occurring hemochromogen containing the same heme as hemoglobin. In these organisms a brown pigment is also found, helicofuscin. *Actineohematin* is a red dye found in the actinae, or sea anemones, and it has been demonstrated to contain heme (McMunn). *Echinochrom* is a brown respiratory pigment found in sea urchins, and may be a heme pigment. Turacin, shown in the tabulation on page 498 under aetioporphyrin I, is a copper salt of uroporphyrin and is found in bird's feathers (Fischer).

* Philippi isolated a pigment group from the hemocyanin of *Helix pomatia* supposed to correspond to hematoporphyrin and giving strong pyrrole reactions. Conant has isolated from the alkaline decomposition of *Limulus* hemocyanin, a black material believed to be a prosthetic group of the respiratory protein complex and consisting of a copper salt of a polypeptide and a sulfur-containing amino acid. He was not able to obtain from it any pyrrole. This raises the question whether hemocyanin should be classed here, or with the nonheme pigments. Does the prosthetic group contain both a pyrrole complex and the thiopeptide, or only the latter, or does it differ in different genera? Svedberg has shown that the sedimentation velocity of hemocyanins are quite different for different genera and phyla, and differs in the two genera in question. Morse applied to *Limulus* hemocyanin a qualitative test for hydroxyprolin, which may be classed as a pyrrole derivative, and found a positive reaction.

NONHEME PIGMENTS OF THE MAMMALIAN ORGANISM

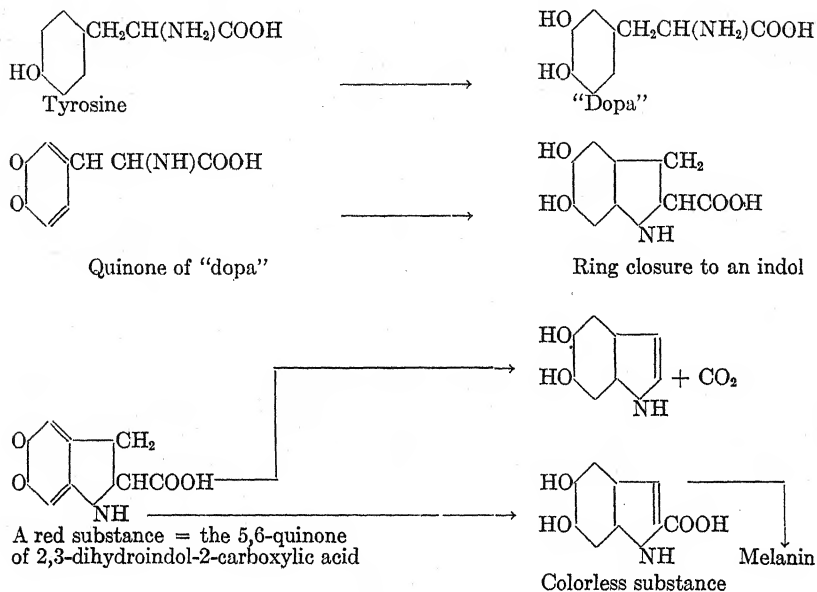
Urochromogen is a colorless substance in freshly voided urine, which gives the Ehrlich diazo reaction of the urine. Alkalis oxidize it to a yellow, **urochrome** (of composition, C 43.09 per cent, H 5.14 per cent, N 11.15 per cent, S 5.09 per cent). On further oxidation, there is formed uromelanin, $C_{47}H_{44}O_{13}N_7S$ (Weiss). The urochrome pigments are supposed to form from the degradation of globin, and possibly from the tryptophan of this protein. **Uroerythrin** or *purpurin* is an amorphous red dye occurring in normal urine and increasing in fever and certain liver diseases. It is iron-free. The constitution is unknown. **Urorosein**, found in pathologic urines, is believed to be formed from a colorless chromogen, indol-acetic acid and has been suggested to be tri-indylmethane.

Indigo or *Indigotin*, occurs in the urine in indiguria. *Indican*, $C_8H_7-C(OSO_3H)=CH$
 $\quad \quad \quad |$
 $\quad \quad \quad NH$ occurs in pathologic urines. *Indigo red* is formed

in some cystitic urines by bacterial action on indoxyl. **Lanaurin** is a pyrrole pigment found in the sweat and urine of sheep and sometimes colors the wool yellow. **Phylloerythrin** or *bilipurpurin*, $C_{34}H_{36}O_6N_4$, is found in oxgall. It is also known as a chlorophyll derivative. It may be derived from chlorophyll of the food of cattle.

Skin Pigments.—**Melanins** are very widely distributed in the animal kingdom. These are the dark dyestuffs of the integument of vertebrates and avertebrates, the pigment of the chorioidea and of melanotic tumors, and the ink secretions of the cephalopods. They are brownish black or black, amorphous, nitrogenous substances, insoluble in most solvents, even the strong acids. Many melanins can be decolorized by ozone or hydrogen peroxide in the presence of light, or by sodium amalgam reduction in alkaline solution. On fusion with potash there is formed a dark-colored melanic acid, soluble in alkalis and precipitable by acids. In the fusion melt there have been found oxalic acid, hydrogen cyanide, ammonia, pyrrole, pyridine and succinic acid. An acidic, phenolic substance, giving a blue ferric chloride reaction is also found. Some have reported indol and scatol.

When a patient has a melanotic tumor, a melanogen is often found in the urine; and this is convertible by oxidizing into the dark-colored melanin. About 1917, it was found that *dioxyphenylalanine* (called "dopa" for short) is transformed into melanin by certain ferments, tyrosinase and "dopa" oxidase. The latter is found in the melanoblasts of the epidermis, for example. The absence of these ferments in unpigmented areas in the integument of albino animals appears significant. Artificial melanin has been made by the action of these ferments on tyrosine *in vitro*. Raper, in the past decade, has made a special study of the melanin reaction. Other catechol derivatives besides tyrosine can be converted to melanins. Raper suggests the following steps; he has isolated some of the intermediate products:



A red pigment, hallochrome, found in the outer skin of *Halla parthenopoea costa* was found by Mazza and Stolfi to be the 5,6-quinone of 2,3-dihydroindole-2-carboxylic acid. It plays a rôle in the oxidation processes of the organism. It will be seen to be identical to the red phase of the "dopa" melanin reaction as postulated by Raper.

Gortner gives as the composition of melanin, C 52.57 per cent, H 7.28 per cent, N 13.43 per cent, S 1.33 per cent, O 25.39 per cent. Raper has shown that amines other than tyrosine can give related oxyindoles. Thus, the pressor amines, adrenaline, tyramine and epinine, can be converted by tyrosinase in this way to indoles and melanins. In the oxidation of "N-methyldopa," Raper finds that a side reaction occurs whereby there is formed adrenaline and adrenalone. "Dopa," itself, also, by a side reaction yields a small amount of pressor substance believed to be amino-acetocatechol.

The fact that adrenaline or its precursors in the body can yield melanins is very suggestive in connection with the observation of extensive brown pigmentation of the skin in pathologies of the adrenal gland (such as Addison's disease). Melanotic tumors are also prolific melanin factories; so that the mechanisms of this particular bio-oxidation process are of considerable interest. Fürth suggests that many amino acids may be condensable to melanin-like bodies. It is possible that urochrome and uromelanin are stages of a similar oxidation of tryptophan. We may count skin melanin as a dyestuff whose light-absorbing properties may have functional, protective significance. Friedheim calls the "red substance" of the melanin reaction an accessory respiratory ferment. The natural melanin of the epidermis of

fishes and other lower forms is often contained in melanophore cells which are contractile. They are caused to contract by sympathicomimetic amines, such as adrenaline. There may be some connection here with the fact that a melanin can form from such amines.

Rhodopsin (the Visual Purple of the Retina).—In the rods of the retina, not only of mammals but of lower forms, there is found the visual purple, concerned in the reception of light by the eye. The chemical nature of this photochemically active dye is unknown. It is extractable from the retina with a solution of bile salts. It is bleached by light. Apparently, it is a fleeting stage of oxidation of some labile chemical. It cannot be kept *in vitro*. The environment bathing the retina, the vitreous humor, is a strongly buffering medium. It has been shown that "dopa" oxidase is present in the retina and rhodopsin is perhaps formed by the action of "dopa" oxidase on a substrate analogous to "dopa." This would place the visual purple in the class of the intermediates of the melanin reaction.

On the other hand we must not forget that the night blindness occurring in vitamin A deficiency is apparently due to a failure of regeneration of the visual purple in the absence of vitamin A. Indeed, Wald claims that the visual purple is a reaction product ("retinene") of a protein with vitamin A.

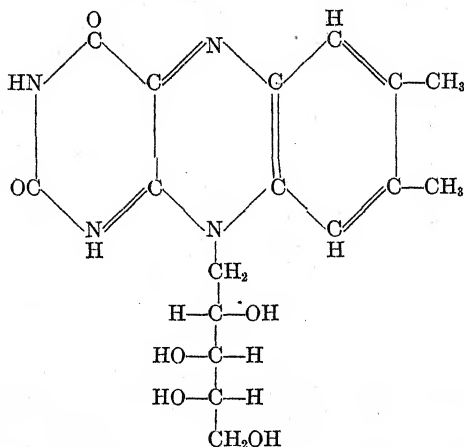
Cytopigments.—**Lyochrome** (ovoflavin; lactoflavin; lactochrome; hefefflavin; cytoflav; vitamin B₂): Richard Kuhn has recently distinguished as lyochrome a nitrogenous water-soluble, orange-yellow, strongly fluorescent pigment, found in egg white, milk whey, and yeast. At first, three separate pigments were reported (ovoflavin, lactoflavin or lactochrome and hefefflavin). Kuhn has now shown that all have identical properties and absorption spectra; and, furthermore, that they all display vitamin B₂ properties and are the most potent sources of vitamin B₂ known. Lactoflavin is assigned the formula C₁₇H₂₀O₆N₄ by Kuhn. Hefefflavin was isolated by Warburg from yeast in the form of a combination with protein, the iron-free respiratory ferment. The hefefflavin is the prosthetic group of the ferment complex.* Both the ferment

* A photoderivative formed by the action of light from the yellow respiratory ferment is given the formula C₁₃H₁₂O₂N₄ by Warburg and he reports that on hydrolysis with barium hydroxide it gives C₉H₁₀O₂N₂. Kuhn states that hydrolysis with sodium hydroxide of the photoderivative of lactoflavin C₁₃H₁₂O₂N₄ gives, with loss of a molecule of urea, C₁₂H₁₂O₂N₂, a carboxylic acid and on heating (decarboxylation) this goes over to C₁₁H₁₂ON₂. Kuhn thinks the C₁₃H₁₂O₂N₄ compound claimed by Warburg is really a mixture of 70 per cent of his compound C₁₂H₁₂O₂N₂ and about 30 per cent of an alkali-soluble product richer in nitrogen which he obtains as a by-product of the alkaline hydrolysis of lumilactoflavin.

Kuhn has recently synthesized both lumilactoflavin and a lactoflavin possessing vitamin B₂ properties. The former he finds to be a 6,7,9-trimethylalloxazin, while the latter has substituted in the 9 position in place of the —CH₃ group the grouping —CH₂(CHOH)₂CH₂OH derived from *l*-arabinose. As there are 8 possible optical enantiomorphs, it is still not certain that the synthetic and the natural product are optically identical, but the synthetic flavin, fed as its tetraacetyl compound, causes marked weight gain in animals on the proper diet at 15 γ daily dose level; hence it displays vitamin activity. No statements have as yet appeared regarding cure or prevention of dermatitis by the synthetic product. The formula of the synthetic pigment is:

and the dye derivable from it can act as vitamin, but only the protein complex can act as ferment. Warburg has recognized the iron-free respiratory ferment spectroscopically as of widespread distribution in nature.

Lipochromes.—These are water-soluble, fat-soluble, nonnitrogenous pigments. Those so far identified have all turned out to be carotinoids of unsaturated polyene nature, with various grouping at the ends of the polyene chains. The lutein of the corpus luteum and lutein of egg yolk, also the lipchrome of nervous tissue, of the blood serum and of the body fat and milk fat, are all carotinoid in nature. The lutein of egg yolk has been shown to be xanthophyll, identical with leaf xanthophyll. Part at least of the lutein of the corpus luteum is caro-



The synthesis is effected by making the amine of *l*-arabinose via the oxime of the sugar and then condensing this with halogen *o*-nitroxylylene; finally in one step the nitro group is reduced to an amine (by means of SnCl_2) and simultaneously alloxan is coupled to the diamine to form the above three-ring structure. Many physical and chemical properties agree with those of lactoflavin from natural sources. Kuhn has also proved a hepaflavin, obtained from liver, to be identical with lactoflavin.

Koshara has identified a dyestuff of normal human urine, a lyochrome, *uroflavin*, which he assigns the formula, $\text{C}_{18}\text{H}_{27}\text{O}_7\text{N}_4$. Ellinger distinguishes a lactoflavin *a* and *b*, also a lactoflavin *c* to which he assigns the formula $(\text{C}_{17}\text{H}_{13}\text{O}_{12}\text{N}_{14})_2$, while the Kuhn compound $\text{C}_{17}\text{H}_{20}\text{O}_6\text{N}_4$ is in his scheme, lactoflavin *d*. The heating of lactoflavin *b* yields lactoflavin *d*, Ellinger claims with splitting off of a purine-like body. The solubilities and crystal forms of the various lactoflavin compounds differ. Flavins have also been isolated from the liver and kidneys of animals and humans and in the eggs of *Myxine glutinosa*.

The above formula makes lactoflavin a pyrimidine derivative. The sugar-like portion has been shown to be essential for the vitamin property. However, one may readily assume that by linking with sugars, polysaccharide structures, or indeed nucleotide or nucleic acid structures, might incorporate this molecule. Whether or not such more complex forms may also possess vitamin properties remains to be seen.

Hugo Theorell (*Biochem. Zt.*, 275, 37 (1934)) has very recently reported that in the yellow iron-free, respiratory ferment there appears to be one mol of phosphorus to one mol of flavin, and that the ferment migrates to the anode at a velocity consistent with a phosphoric ester. He concludes that the active group of the yellow ferment is probably a phosphoric ester, a sort of nucleotide, in which the purine base is replaced by the dimethylalloxazin portion of the lactoflavin.

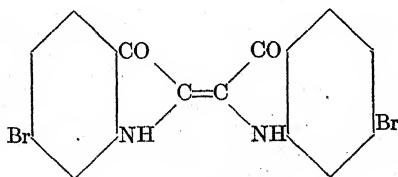
tene, and both of these dyes are also found in serum, nervous tissue, milk fat and body fat. It is suspected that the carotinoids are derived ultimately from vegetable sources. In the case of carotene, this is made evident from the vitamin A deficiency resulting from withdrawal of this provitamin A and the vitamin A itself from the diet. These carotinoids, at least, must come from outside, and ultimately from plant sources. Since provitamin A (carotene) apparently can be converted into vitamin A in the animal liver, the latter may be said to be synthesized in the animal from the carotinoid dye (see Chapter IX).

The yellow pigment of the goose bill is also a "lutein." The pigment of the scales of the gold fish seems to be carotinoid character (Weissberger and Bach). The silver-white of ordinary fish scales is guanine.

The yellow pigments of canary bird feathers and of the feathers of other yellow birds and of the tarsal skin of various birds has been shown to be carotinoid in nature, "canary xanthophyll" and lutein. A red decomposition product in some species colors the feathers or tarsi red. The red epidermal papillae around the eyes of pheasants are said to contain a carotinoid dye resembling the astacin of the lobster (see below).

Nonheme Pigments of Lower Organisms.—A very heterogeneous group of substances must be collected under this heading. Many are of totally unknown constitution and only their absorption spectra, or indeed, only the methods of isolating them serve to characterize them. Only a few of the better characterized substances can be mentioned.

Royal purple (Tyrian purple; 6,6'-dibromindigo) is obtained from *Murex brandaris* and was one of the purples of the ancients. It was synthesized in 1909 by Paul Friedlander. The formula is



Punizin is a purple dye formed by light and air from a colorless chromogen found in the secretions of *Murex trunculus* and of *Purpura lapillus*. This is also a purple of the ancients. The structure is unknown.

Crustaceorubin (zoönerythrin; tetra-erythrin; vitellorubin) is a brown-black pigment in crustacea. This is found in lobster shells and lobster eggs, for example, and also in the eggs of the spider crab, *Maja squinado*, in crayfish, spiny lobster, and certain other crabs. Kuhn has shown it to be a chromoprotein which yields, on heating, red esters of a dyestuff, astacin, $C_{27}H_{32}O_3$. This carotinoid can take up ten mols of hydrogen on catalytic hydrogenation. An astacin ester occurs

uncoupled to protein in the red hypodermis of the lobster. This crustaceorubin is the only known example of a carotinoid serving as a prosthetic group, coupled to a protein.

Among red dyes may be mentioned carmic acid, $C_{22}H_{20}O_{13}$, a glucoside, the red pigment of the cochineal insect. The aglucone, carmine red, is $C_{11}H_{12}O_7$, an anthracene derivative. On oxidation this yields a substituted naphthoquinone. Kermes, an insect, contains a brick-red coloring matter. Kermesic acid is 1,3,4,6-tetraoxy-2-aceto-8-methylanthraquinone. In sponges occur red floridines; in crinoids, red antedonine. Aplysiopurpurin is a purple red dye in the glands of *Aplysia*, marine gastropods.

Uranidines, red or yellow in color, are found in sponges, corals, medusae, holothurians and worms. These are suggested to be related to melanins and to be formed by the aid of a tryptophanase-like oxidase.

Yellow purin pigments are known, such as the lepidopteric acid or leucopterin, isolated by F. G. Hopkins from butterfly wings. H. Wieland has shown this to be a condensation product of three mols of guanidine and to have the formula $C_{19}H_{19}O_{11}N_{15}$. He also has found a related xanthopterin, $C_{19}H_{19}O_7N_{15}$. These pigments are found in the integument (hypodermis) of wasps and hornets as well as in the wings of butterflies.

Among blue dyes may be noted cyanein in medusae and cyanocrystalline, a blue dye of the integument of decapods. Heliotis indigo apparently identical with indigo, has been found in the shells of the mollusk, *Heliotis californiensis*. A blue dye is known in certain fishes, such as *Chenilabrus pavo*.

Green dyes of unknown constitution have been reported in worms and insects. The sepia of the octopus is a melanin, as noted above.

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REFERENCES

- Blood and Bile Pigments.*
 Reinbold, B., von, in Abderhalden's *Biochemisches Handlexikon* (1911), 6, 188-292.
 Küster, W., in Abderhalden's *Handbuch der biologischen Arbeitsmethoden* (1922), Abt. I, Teil 8, 201-350.
 Küster, W., in Abderhalden's *Biochemisches Handlexikon* (1923), 10 (3 *Ergänzungsband*), 1-88, 916-931.
 Fischer, H.: (Pyrrole dyes) in Oppenheimer's *Handbuch der Biochemie* (1924), 1, D, IV, 351-404.
 Müller, F., and Biehler, W.: (Respiratory dyes) in Oppenheimer's *Handbuch der Biochemie* (1924), 1, D, V, 405-476.
 Fürth, O.: (Urinary dyes) in Oppenheimer's *Handbuch der Biochemie* (1924), 1, F, III, 941-943.
 Treibs, A.: (Cellular dyes containing iron) in Oppenheimer's *Handbuch der Biochemie* (1924), 1, B, III, 104-116.
 Fischer, H.: (Blood and bile dyes) in Oppenheimer's *Handbuch der Biochemie*, (1924), *Ergänzungsband*, A, III, 72-104.
 Fischer, H.: *Ber.*, 60, 2611-2651 (1927); *Naturwissenschaften*, 17, 611-617 (1929).
 Fischer, H., and Neumann, F. W.: *Annual Review of Biochemistry* (1932), 527-535.
 Mirsky, A. E., and Anson, M. L.: (Animal pigments), *Annual Review of Biochemistry* (1932), 535-550.
 Haurowitz, E.: *Fortschritte der Biochemie* (1932), Teil II, 87-99.

- King, H.: (Pyrrole pigments, heme compounds, etc.) in *Annual Reports on the Progress of Chemistry* (The Chemical Society, London, 1932), 29, 209-219.
- Gardner, J. A., and Buchmaster, G. A.: In Allen's *Commercial Organic Analysis* (1933), 10, 1-118.
- Maurer, H.: In Abderhalden's *Biochemisches Handlexikon* (1933), 14, (7 *Ergänzungsband*), 340-797.
- Seidel, W., and Fischer, H.: *Z. physiol. Chem.*, 214, 145-172 (1933).
Melanins, Lipochromes, Dyestuffs of Lower Organisms.
- Samuely, F.: In Abderhalden's *Biochemisches Handlexikon* (1911), 6, 293-377.
- Samuely, F., and Strauss, E.: In Abderhalden's *Handbuch der biologischen Arbeitsmethoden* (1922), Abt. I, Teil 8, 731-790.
- Fürth, O.: In Oppenheimer's *Handbuch der Biochemie* (1924), 1, F, IV, 944-960.
- Pryde, J.: *Recent Advances in Biochemistry* (1928), Chapt. X.
- Duliere, W. L., and Raper, H. S.: (Melanins), *Biochem. J.*, 24, 239-249 (1930).
- Conant, J. B., Chow, B. F., and Schoenbach, E. B.: (Hemocyanin), *J. Biol. Chem.*, 101, 463-473 (1933).
- Morse, Withrow: *J. Biol. Chem.*, 100, 373-377 (1933).
- Wieland, H., Metzger, H., Schöpf, C., and Bülow, M.: (Leucopterin-xanthopterin), *Ann.*, 507, 226-265 (1933); Schöpf, C., and Bülow, M.: *Ann.*, 507, 266-296 (1933).
- Miescher, G.: (Visual purple), *Klin. Wochschr.*, 1, 173-174 (1922).
- Wald, G.: *Nature*, 134, 65 (1934).
- Kuhn, R., et al.: (Lactoflavin chemistry), *Ber.*, 67, 1460-1462, 1932-1936, 1939-1941, 2084-2085 (1934).
- Brockmann, H., and Völker, O.: (Bird feather pigments), *Z. Physiol.*, 224, 193-215 (1934).

CHAPTER XIX

OXIDATIONS AND REDUCTIONS

FROM the purely physical viewpoint, life is dependent on energy interchanges; and these are associated with the syntheses and decompositions of the constituents of living matter, processes that involve oxidations and hydrolyses* and their reverse operations. Green plants are adapted for the direct utilization of certain portions of the radiant energy emitted by the sun; and the latter thereby stands, in the last analysis, as the ultimate source of energy for most of the living matter on the earth. Although the mechanism of the energy transformation in chlorophyll-containing plants is not yet clear in many particulars, it is quite certain that the process involves a reduction of CO_2 of the air leading to the ultimate formation of formaldehyde and its polymers: Sugar, starch and cellulose. There is another class, the autotrophic organisms, first recognized by Winogradsky, which are not immediately dependent upon preformed organic matter but can utilize the energy liberated by oxidation of inorganic substances, like sulfur, to reduce CO_2 and thus raise the chemical potential of carbon compounds. In general, however, living cells obtain their necessary energy through stepwise oxidative decompositions of preformed organic matter accomplished by mechanisms which will be discussed below.

Energetics.—The origin and significance of animal heat was first clearly shown by Lavoisier when, with Laplace, he measured by means of his ice calorimeter the heat outputs of a guinea-pig and of glowing charcoal and related them to the corresponding outputs of CO_2 . During the century following this fundamental experiment, there grew the ideas upon which the twin sciences of thermochemistry and animal calorimetry have developed; and careful experiments have shown that in the complete oxidation of a foodstuff, like carbohydrate, to CO_2 and H_2O , the heat output as measured in the bomb calorimeter is in very close agreement with that produced by a resting living creature in the modern animal calorimeter. Thus is established the basic principle that the animal is like a machine capable of transforming energy ultimately into heat: A principle that finds its applications in nutrition and dietetics. We know, however, that the animal body is not in any direct sense a heat engine. It utilizes its energy supply for chemical syntheses and transformations (including solution, ionization, surface effects, osmotic pressure) with heat as a by-product. In short, it transforms chemical energy into work and heat in accordance with the first law of thermodynamics, the law of the conservation of energy.

* Many of the hydrolytic processes involve relatively small energy exchanges; but there are others, *e. g.*, the splitting of creatine phosphate and arginine phosphate, in which the heat of reaction is quite considerable.

Although it will be necessary to introduce certain thermodynamic considerations in the development of the subject of oxidations and reductions, it is impossible to give in brief compass an adequate presentation of the underlying principles and derivations. For these, the reader should consult such texts as those by Lewis and Randall,¹ or Macdougall.² We shall restrict ourselves here to the mere statement of certain important concepts and principles and some of the deductions applicable to the present discussion.

One of the properties of any material system is its content of energy which can be increased or decreased in various ways, the change in internal energy being the difference between the energies of the final and initial states.

When a system loses energy by radiation or thermal conduction, we may say that it is giving up heat; and when it loses energy by operating against external forces, usually mechanical, it is doing work.

The most general statement of the first law is contained in the equation

$$E_B - E_A = \Delta E = q - w \dots \dots \dots (1)^*$$

That is, the increase in internal energy, ΔE , of a system as it changes from state *A* to state *B*, is equal to q , the heat absorbed from the surroundings, less w , the work done by the system upon the surroundings. If in any process the pressure, P , is kept constant and the only form of work is that due to change in volume, ΔV , of the system, then the work done is

$$w = P\Delta V \dots \dots \dots (2)$$

Consequently, the heat absorbed is

$$q = \Delta E + P\Delta V \dots \dots \dots (3)$$

and it is obvious that the heat absorbed depends only on the initial and final states of the system. (If the volume is kept constant, the last term in equation 3 equals zero.)

The relation given in equation 3 is useful in thermochemistry because most calorimetric work is done at constant pressure, in which case, the heat change is called ΔH , and we have

$$\Delta H = q = \Delta E + P\Delta V \dots \dots \dots (3a)$$

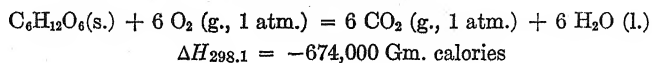
This leads to the definition of another quantity, *viz.*:

$$H = E + PV \dots \dots \dots (4)$$

where H is called the heat function or heat content of a system. No attempt is made to give this an absolute value; but its change or

* In this discussion, the Lewis and Randall¹ notation is used except as to the convention regarding the sign of electrode potentials, in which case the convention of *International Critical Tables* is followed.

increment, ΔH , is measurable and is the thermochemical quantity usually determined. Thus the process, referred to 25° ,



represents the combustion of 1 mole of solid glucose with oxygen gas to gaseous CO_2 and liquid water, all at one atmosphere pressure. When the final products are brought to the temperature of the initial system (absolute temperature $= T = 298.1^\circ$) the net increase in heat content, ΔH , of the products is $-674,000$ Gm. calories, a minus quantity because the heat is liberated in the process. It can be demonstrated that the quantity of heat absorbed or evolved when a system goes from one state to another is independent of the nature of the intermediate stages so long as the process is carried out either at constant pressure or constant volume.

It was believed at one time that the heat evolved is a direct measure of chemical affinity or the driving force tending to determine the direction of a chemical reaction. The general validity of this is, however, challenged by the fact that some reactions may proceed spontaneously while accompanied by the absorption of much heat.

It might also be assumed that $-\Delta H$ presents the limiting amount of work obtainable under conditions of maximum efficiency from a chemical reaction, but this may or may not be true depending on the nature of the process. The scope of the First Law is limited to the quantitative overall energy content of a system undergoing change, but it has nothing to say about the direction of the process or about the convertibility of the energy that is liberated. One must turn to the Second Law which defines the limitations that govern the transformation of heat into work, of one form of energy to another. It sums up our experience that all systems tend to approach an ultimate state of rest or statistical equilibrium and in a measure lose their capacity for spontaneous change; *i. e.*, that all actual processes are irreversible to a greater or less degree. The Second Law is thus known as the law of the degradation or dissipation of energy, or the law of the increase of entropy. Entropy has the same dimensions as heat capacity. Entropy increase, ΔS , is defined by the equation

$$\Delta S = \frac{q}{T} \dots \dots \dots (5)$$

and may be considered the measure of irreversibility of a process. An irreversible process is one in which the restoration of a system to its original state cannot be accomplished except at the expense of some permanent change elsewhere. It is evident that the total entropy is unchanged in the ideal, reversible process.

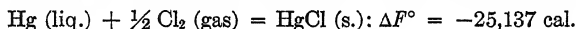
We may now combine the two laws to define F , the free energy

$$F = H - TS \dots \dots \dots (6)$$

It represents the maximum work which is obtainable from a process

occurring at constant temperature and pressure and available for useful work. F and H are not equal except when the entropy is the same in the initial and final states; and since this defines a reversible process, we can say that the latter is capable of furnishing maximum useful work. It is the free energy change which enables one to decide whether a process may take place spontaneously or not; and its magnitude, in a chemical reaction, is considered a measure of the available chemical energy that may be utilized. When ΔF is negative, the process may occur spontaneously; when $\Delta F = 0$, a state of equilibrium exists; and when ΔF is positive, the process can occur only if energy from some outside source is added to the system. It should be emphasized that thermodynamics is not concerned with the time variable and therefore has very little to say about the kinetics of a process. It does not deal with mechanisms except to distinguish between those that are probable and those that are not.

In actual calculations and determinations of the free energy change in a chemical reaction, it is advantageous to select as a convenient point of reference some standard state for each component, usually at atmospheric pressure and 25° C. When a compound is formed from its elements in their standard reference states, for example,



the ΔF° , the standard free energy change, is called the free energy of formation of the compound. ΔF° is an extensive property which may be added algebraically to yield the net free energy change of a process.

Furthermore, the activity of a substance in the chosen standard state is taken as unity, *i. e.*, $a^\circ = 1$. Activity is defined by the equation: $RT \ln a = F$, where R is the gas constant (1.9869 cal.₁₅, deg.⁻¹, mole⁻¹), T is the absolute temperature and \ln symbolizes natural logarithms. It is the "corrected concentration" or "thermodynamic concentration" as represented by the behavior of an ideal, attenuated gas. If a substance is transferred reversibly from its standard state, a° , to some other state a , the free energy change involved is defined by the equation

$$\Delta F = \Delta F^\circ + RT \ln a - (\Delta F^\circ + RT \ln a^\circ) = RT \ln \frac{a}{a^\circ}$$

This is a special, simple case. For a process involving a number of components, the same equation applies with certain modifications. For instance, consider a general reaction in which m mols of substance M, etc., produce r mols of R, etc., as represented by



It can readily be shown for this case that

$$\Delta F = \Delta F^\circ + RT \ln \frac{a_R^r \cdot a_S^s \cdot \dots}{a_M^m \cdot a_N^n \cdot \dots} \quad \dots \dots \dots (7)$$

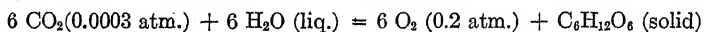
The fraction in the last term is known as the activity quotient of the reaction.

Since $\Delta F = 0$ at equilibrium, equation 7 then resolves to

$$\Delta F^\circ = -RT \ln K. \dots\dots\dots (7a)$$

where K is the equilibrium constant, the value of the activity quotient when the system is at equilibrium. This is the thermodynamic equilibrium constant which is identical with the mass law equilibrium constant when concentrations and pressures are substituted for activities.

Equations 7 and 7a are very useful tools because they permit the calculation of ΔF for any desired activities of the reacting substances. For example, the synthesis of sugar in photosynthesis may be written (using pressures actually occurring):



Calculation shows that this process requires an input of $\Delta F_{298.1} = 708,900$ calories; and when the components are in their standard states, $\Delta F^\circ_{298.1} = 685,000$ calories.³ Examples of the employment of equations 7 and 7a in the calculation of free energies in chemical and biological processes will be found in the publications of Burk⁴ and Wilson and Peterson.⁵

Potential.—Work may display itself kinetically or it may be stored as "potential" energy. Now, energy, in its dimensions, may be considered as a composite of two factors: Intensity and capacity. Thus, mechanical energy is measurable as force times distance (or pressure times volume); thermal energy, by temperature times gram calories; electrical energy by volts times coulombs; chemical energy, by chemical potential times mass. It is the intensity factor which is of peculiar significance in determining the direction of a process. Heat tends to flow from regions of higher to regions of lower temperature; electricity, from systems of higher to systems of lower electrical potential; chemical energy, from systems of higher to systems of lower chemical potential. The capacity factor merely determines the extent to which the energy may flow before equilibrium sets in. In a chemical process, we may regard the chemical potential, or, more strictly, the potential difference as the driving element of the reaction in the same sense that the net height of a waterfall is the driving force of the mechanical work which it can perform.

The Reversible Galvanic Cell.—It was stated that all actual processes are more or less irreversible; but in certain galvanic cells the processes of charge and discharge at constant temperature and pressure as measured by a very sensitive galvanometer can be so delicately balanced by a suitable potentiometer system that, for all practical purposes, the cell processes may be considered reversible. Since the criteria for thermodynamic reversibility are satisfied in such a process, the total entropy change is zero; and since the electrical potential as measured potentiometrically is obtained under conditions of maximum

work, we have here a direct measure of the free energy change in the cell reaction. Thus, because the reversible electrochemical cell, where applicable, is an elegant and accurate means for measuring free energy changes, it is convenient to formulate relationships in terms of electrical energy: Electromotive force (volt) and amount of electricity per gram equivalent of any ion (Faraday).

The *Faraday* (designated by F) is taken as a basic constant by *International Critical Tables* and given the value of 96,500 coulombs (abs.) per gram equivalent. Measurements of differences in electromotive force (designated by E) are made in terms of the volt. Thus, electrochemical work done by a cell may be written, $-w' = -NEF$, where N represents the number of equivalents exchanged, and the minus sign indicates, according to convention, that the work is expended by the reacting system. We may therefore write for the free energy change in a reversible galvanic cell at constant temperature and pressure

$$\Delta F = -NEF \dots \dots \dots (8)$$

$$\Delta F^\circ = -NE^\circ F \dots \dots \dots (8a)$$

Combining equations 7, 8 and 8a

$$\Delta F - \Delta F^\circ = NE^\circ F - NEF$$

$$E^\circ - E = \frac{RT}{NF} \ln \frac{a_R^\tau \cdot a_S^g}{a_M^m \cdot a_N^n} \dots \dots \dots (9)$$

At equilibrium, $E = 0$, and the activity quotient equals K , the equilibrium constant. Therefore, the "equilibrium" potential

$$E^\circ = \frac{RT}{NF} \ln K \dots \dots \dots (9a)$$

defines the electromotive force (potential difference) of a reversible electrochemical cell reaction at constant temperature and pressure, in terms of the activities of the components. In actual practice, it often happens that the data on activities are lacking; and it is then necessary to use approximation values as represented by (stoichiometrical) concentrations. This is the case with most of the systems which will be considered here.

The foregoing rough sketch of the elementary concepts of thermodynamics is necessary in order that the student of oxidation-reduction may more fully appreciate the fundamental significance of the phenomena that will now be discussed.

Reversible Oxidation-reduction Cells.—The electrode potential difference between the poles of a galvanic cell is measured in terms of the international volt; but it is necessary, in addition, to establish some fixed point to which various potentials may be referred, in analogy to the arbitrary zero point on the Centigrade temperature scale. The electrode potential of hydrogen at atmospheric pressure (unit activity)

against hydrogen ions at unit activity in aqueous solution is taken to be zero at all temperatures. This refers to the hydrogen: hydron half-cell which constitutes the so-called "normal hydrogen electrode." The process in this half-cell may be written



where ϵ represents the electron equivalent or F coulombs of negative electricity.

Let us next combine this reference system with any other half-cell, *e. g.*, the ferrous: ferric system, $\text{Fe}^{++} \rightleftharpoons \text{Fe}^{+++} + \epsilon$. The whole reaction is:



Then, the electromotive force of this reversible process is given by

$$E = E^\circ + \frac{RT}{F} \ln \frac{a_{\text{H}} \cdot a_{\text{Fe}^{+++}}}{a_{\text{H}^+} \cdot a_{\text{Fe}^{++}}} \dots\dots\dots (12)$$

and, since the standard electromotive force E° , of the cell, equals the sum of the single potentials E°_{H} and E°_{Fe}

$$E = E^\circ_{\text{H}} + E^\circ_{\text{Fe}} + \frac{RT}{F} \ln \frac{a_{\text{H}} \cdot a_{\text{Fe}^{+++}}}{a_{\text{H}^+} \cdot a_{\text{Fe}^{++}}} \dots\dots\dots (12a)$$

But, by definition $E^\circ_{\text{H}} = 0$ when a_{H} and a_{H^+} each equals unity, therefore

$$E_{\text{h}} = E^\circ_{\text{Fe}} + \frac{RT}{F} \ln \frac{a_{\text{Fe}^{+++}}}{a_{\text{Fe}^{++}}} \dots\dots\dots (13)$$

where E_{h} is used instead of E in order to signify that the potential is referred to E°_{H} , the standard hydrogen electrode potential. Equation 13 is known as the Peters equation.¹²

At this point we shall define the *oxidation* of a substance as its loss of electrons, loss of hydrogen or addition of oxygen; *reduction* is the converse of these processes.

In general, for any system involving N equivalents, the half-reaction may be written



and, when combined as above with the normal hydrogen half-cell, the potential of the system is

$$E_{\text{h}} = E^\circ + \frac{RT}{NF} \ln \frac{a_{\text{Oxidant}}}{a_{\text{Reductant}}} \dots\dots\dots (15)$$

It will be noted that $E_{\text{h}} = E^\circ$ when the activity quotient equals unity, other conditions, especially pH , remaining constant. Consequently, it becomes possible to express relative oxidation-reduction intensities in terms of electrode potential.⁶ This is illustrated in Fig.

35. The distinct slopes of curves *A* and *B* are determined by the values of *N* in equation 15. The position of a system on the potential scale depends on the constant, E° . Note that the curves become asymptotic at each end where the electrode potentials tend to become more and more indefinite, and that consequently there is no meaning to the elec-

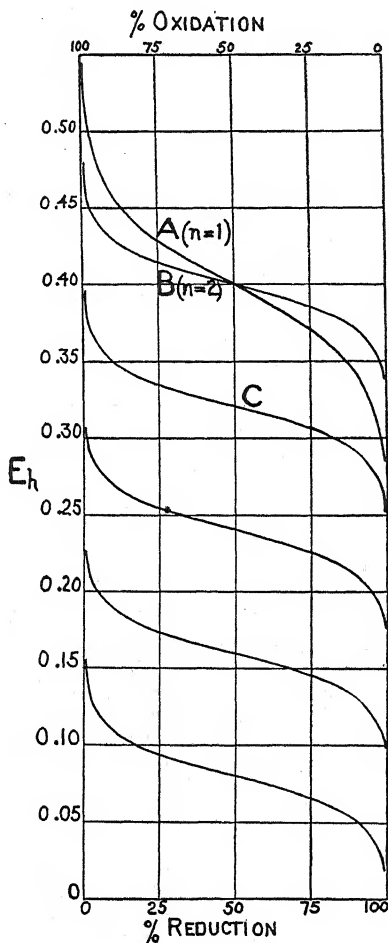


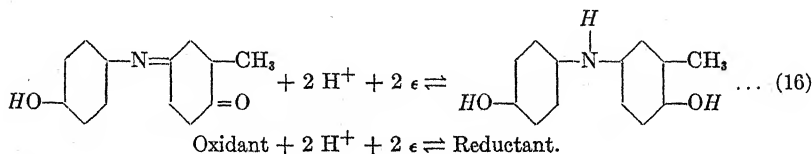
Fig. 35.—Relation between percentage reduction and E_h in systems having different E° values. (W. Mansfield Clark, *U. S. Public Health Reports*, Vol. 38.)

trode potential of a pure oxidant or a pure reductant. Finally, if we have a system at the midpoint ($E_h = 0.32$) of curve *C*, it will tend to oxidize any system having an E_h value more negative than 0.32, and reduce any system more positive.

It will be noted that a curve represented by equation 15 is flattest near the midpoint (at 50 per cent reduction); that is, the potential

suffers less change near the midpoint than it does near the (asymptotic) ends for equal small changes in the per cent reduction. This stabilization of the oxidation-reduction potential is analogous to buffering action in acid-base systems, and is called *poising action*.⁶

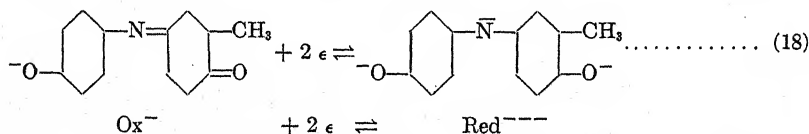
Effect of pH.—To illustrate another important point, consider the system *o*-cresol indophenol: leuco-compound. The isolated compounds differ by two hydrogen atoms; and the same would be essentially the case in strongly acid solution where hydron dissociation would be suppressed. The oxidation-reduction can be written:



The electrode equation would be (assuming $a_{\text{H}^+} = 1$ for purposes of simpler illustration):

$$E_h = E^\circ + \frac{RT}{2F} \ln \frac{a_{\text{Oxidant}}}{a_{\text{Reductant}}} \quad (17)$$

Now imagine the compounds in a hypothetical, extremely alkaline solution where all dissociable hydrions (indicated by *H* in the structural formulas) have been stripped off. The reaction would then be:



$$\text{Therefore, } E_h = E^\circ + \frac{RT}{2F} \ln \frac{a_{\text{Ox}^-}}{a_{\text{Red}^{--}}} \quad (19)$$

As would be expected, equations (17) and (19) are identical in form, but in the latter the free energy exchange involves, in addition, the energies of hydrion dissociation in both oxidant and reductant. That is to say, as we carry the indophenol system from one end of the acidity scale to the other, each dissociable hydrion will dissociate in the order of its "strength" as an acid as measured by its dissociation constant. In the case of weak electrolytes, such as we are considering, with each step in dissociation the free energy of the parent compound will be changed by an amount proportional to $RT \ln K$, where K is the dissociation constant of the particular hydrion involved. It follows, therefore, that a complete formulation of the oxidation-reduction must include the participation of hydrions (taking into account also their energies of "dilution").

Hence, the general equation 15 must be amplified to:

$$E_h = E^\circ + \frac{RT}{nF} \ln \frac{a_{\text{Ox}}}{a_{\text{Red}}} + (\text{function of } a_{\text{H}^+} \text{ and } K_1, K_2 \dots) \quad (20)$$

For example, the corresponding equation for the *o*-cresol indophenol system in the experimentally attainable regions of *pH* is:

$$E_h = E^\circ + 0.03 \log \frac{[\text{Ox}]}{[\text{Red}]} - 0.03 \log \frac{K_o + [\text{H}^+]}{K_{r1}K_{r2}[\text{H}^+] + K_{r1}[\text{H}^+]^2 + [\text{H}^+]^3} \dots (21)$$

where K_o = dissociation constant of phenolic hydrion in oxidant
 K_{r1} = " " " " same phenolic hydrion in reductant
 K_{r2} = " " " " phenolic group created by reduction

$$0.03 \log = \frac{RT}{2F} \ln \text{ at } 30^\circ \text{ C.}$$

Data on activities are lacking; therefore equation 21 is written in terms of concentrations (indicated by brackets) and mass-law dissociation constants.

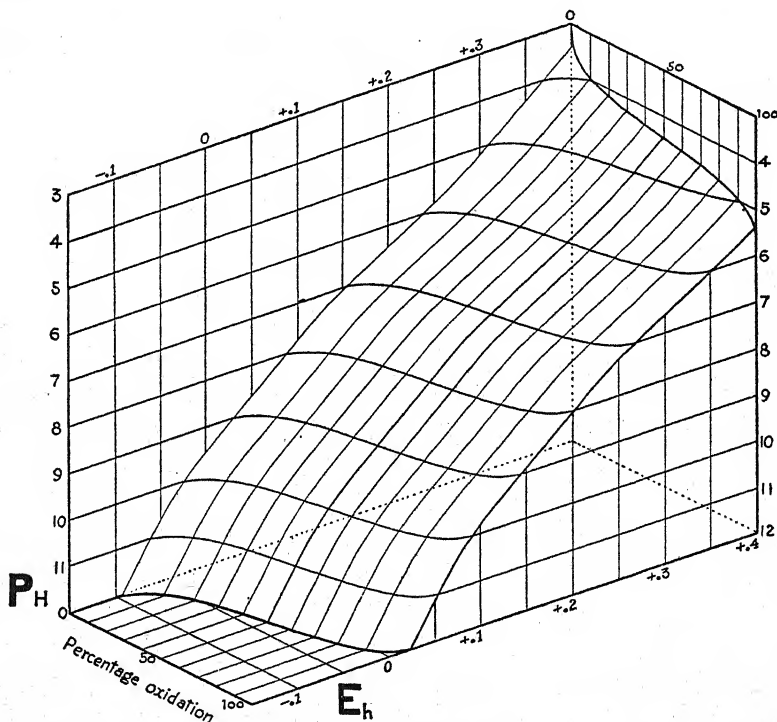


Fig. 36.—Three-dimensional surface relating the potential of a reversible oxidation-reduction (*viz.*, indophenol) system to changes in *pH* and percentage oxidation. (Cohen, Gibbs and Clark, *U. S. Public Health Reports*, Vol. 39.)

An interesting consequence of theoretical importance is the fact that the process of reduction may create acidic properties or destroy basic properties of the reactants.

Equation 20 describes a three-dimensional surface such as is shown in Fig. 36; the coordinates are E_h , *pH* and percentage oxidation.

In the E_h : percentage oxidation plane this surface presents a uniform S-shaped intercept; in the E_h : pH plane the intercept is a more or less complicated curve with bends corresponding to the various hydron dissociations. Therefore a two-dimensional plot of E_h vs. pH will be useful for comparing one system with any others. Any E_h : pH intercept may be chosen, but it is advantageous to select the one running through the S-curves at their midpoints where the ratio of oxidant to reductant is unity. Then, if we write the equation for the S-shaped intercept,

$$E_h = E^\circ + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]}$$

when the ratio = 1, $E_h = E^\circ$. We shall designate by E'_0 the potentials of the midpoints of all systems except those at unit hydron concentration where E° applies.

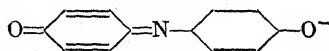
Only a few of the many systems thus mapped out can be mentioned here. For the others, reference should be made to the monographs by Wurmser,⁷ and by Michaelis⁸ and the papers by Clark and Cohen^{6, 10} and others quoted in this chapter. The systematic studies of Clark and coworkers have resulted in the development of a series (still incomplete) of indicators of oxidation-reduction analogous to acid-base indicators. Their application in cases where an electrode cannot be used is self-evident.

The accompanying table presents a tentative list of available indicators selected by Cohen¹⁶ as suitable. Several new dipyrindyl deriva-

A SELECTION OF REVERSIBLE OXIDATION-REDUCTION INDICATOR SYSTEMS

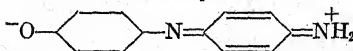
E'_0 values are those at pH 7.0. E'_0 and E° are in volts referred to the normal hydrogen electrode. All the data were determined at 30° C. Names and type structures are those of the respective oxidants. In the literature references, data on E'_0 values at other pH numbers can be found.

Indophenols^{6, 21}

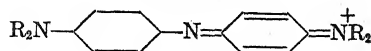


| | E'_0 | E° |
|--|--------|-----------|
| Phenol- <i>m</i> -sulfonate indo- 2, 6-dibromophenol. | 0.273 | 0.6906 |
| <i>m</i> -Chlorophenol indo- 2, 6-dichlorophenol. | 0.254 | 0.6919 |
| Phenol- <i>o</i> -sulfonate indo- 2, 6-dibromophenol. | 0.242 | 0.6834 |
| <i>o</i> -Chlorophenol indophenol. | 0.233 | 0.6627 |
| 2, 6-Dichlorophenol indophenol. | 0.217 | 0.6684 |
| 2, 6-Dichlorophenol indo- <i>o</i> -cresol. | 0.181 | 0.6394 |
| 1-Naphthol-2-sulfonate indo- 2, 6-dichlorophenol. | 0.119 | 0.5630 |

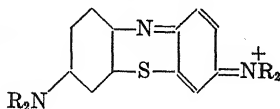
Amino-indophenols²²



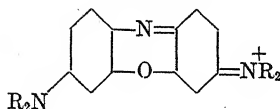
| | E'_0 | E° |
|--|--------|-----------|
| Phenol blue. | 0.225 | 0.677 |
| <i>m</i> -Toluylene diamine-indophenol. | 0.125 | 0.567 |

*Indamines*⁴²

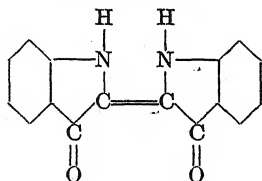
| | E'_0 | E° |
|--------------------------|--------|-----------|
| Bindshedler's green..... | 0.224 | 0.680 |
| Tolylene blue..... | 0.115 | 0.601 |

*Thiazines*⁶

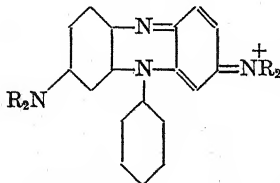
| | E'_0 | E° |
|---------------------|--------|-----------|
| Lauth's violet..... | 0.062 | 0.563 |
| Methylene blue..... | 0.011 | 0.532 |

*Oxazines*²⁴

| | E'_0 | E° |
|----------------------------------|--------|-----------|
| Cresyl blue..... | + .047 | 0.583 |
| Methyl Capri blue..... | -.061 | 0.477 |
| Ethyl Capri blue..... | -.072 | 0.540 |
| Nile blue.HSO ₄ | -.122 | 0.406 |

*Indigo sulfonates*⁶

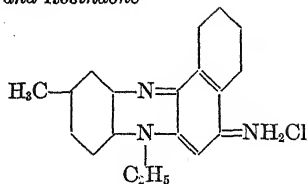
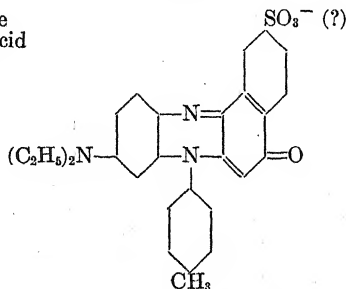
| | E'_0 | E° |
|----------------------------|--------|-----------|
| Indigo tetrasulfonate..... | -.046 | 0.365 |
| Indigo trisulfonate..... | -.081 | 0.332 |
| Indigo disulfonate..... | -.125 | 0.291 |
| Indigo monosulfonate..... | -.159 | 0.262 |

*Safranines*²⁵

| | E'_0 | E° |
|---|--------|-----------|
| Phenosafranine (Rowe 840)..... | -.252 | 0.280 |
| Tetra-ethylphenosafranine (Rowe 847)..... | -.254 | 0.355 |
| Dimethylphenosafranine (Rowe 842)..... | -.260 | 0.286 |
| Tetramethylphenosafranine..... | -.273 | 0.288 |
| Safranine T (Rowe 841)..... | -.289 | 0.235 |

Rosinduline and Rosindone

Induline scarlet (Rowe 827)

Sulfonated rosindone
(position of sulfonic acid
group uncertain)

| | E'_0 | E° |
|--------------------------------------|--------|-----------|
| Rosinduline scarlet (Rowe 827) | -0.296 | 0.047 |
| Sulfonated rosindone | -0.380 | 0.25 |

tives (viologens) have recently been described by Michaelis and Hill.¹⁷ These lie in the region of the hydrogen potential at pH 7. The factors involved in the proper use of oxidation-reduction indicators are somewhat complicated by the varied chemical nature of the compounds. Some of the practical and theoretical aspects of the use of the indicators are discussed by Cohen.¹⁶ The advantage of the mapping out of the various oxidation-reduction systems will be apparent in an example selected from the work of Ball and Chen.⁹

Figure 37 shows the relations of the E'_0 : pH curves for several interesting systems, which, for convenience, are usually, except for special emphasis, designated by the names of the oxidants. The hydrogen electrode (H^+ : H) system is shown in the lower left-hand corner. This line has a slope of -0.0601 at 30°C ., that is $-\Delta E'_0/pH = 0.0601$ volt. The ferricyanide system shows no variation of potential with pH *within the indicated region of pH*. The benzohydroquinone system has the same slope as the hydrogen electrode down to nearly pH 9 before dissociation causes the curve to bend. The methylene blue system has, in acid regions, a slope of 0.09 which transforms to 0.03 in alkaline regions. The other curves show their individual trends, and it is at once obvious that a system may be "oxidizing" or "reducing" depending on (a) its position relative to other systems on a diagram of potential such as this and (b) on the pH. The latter point is made clear by comparing systems D and G in Fig. 37. At pH values less than 6, the oxidant of D should oxidize the reductant of G, while at $pH > 6$ G-oxidant should oxidize D-reductant.

We may now examine the observations on reducing conditions in

cell suspensions and the cell interior in order to better appreciate biological oxidation-reductions and potential as a factor in their mechanisms.

Reduction Potentials in Cultures and Cells.—In 1861 Pasteur discovered anaerobic bacteria which can grow in the "absolute" absence of oxygen. When confronted by Gunning in 1878 with the objection that organisms must have some free oxygen for growth, Pasteur referred to his experiments with indigo carmine which was kept reduced

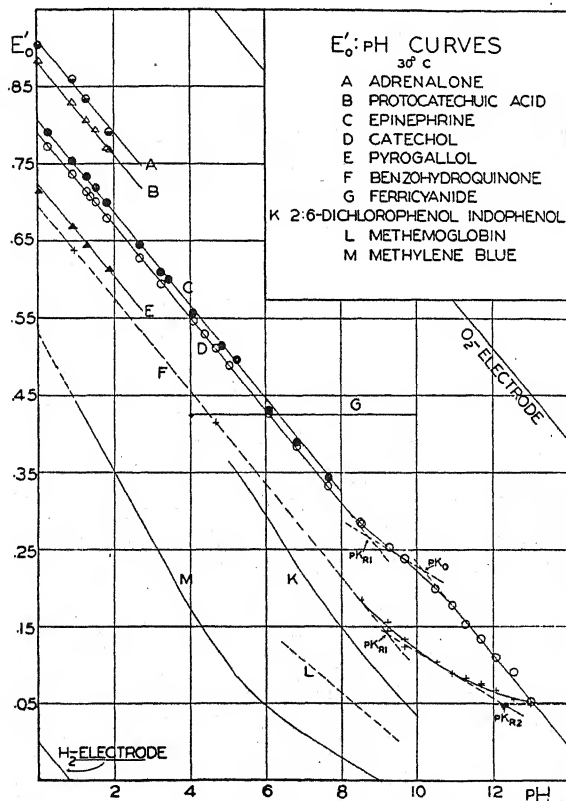


Fig. 37.—Relation to E'_0 to pH. (Ball and Chen, *Jour. Biol. Chem.*, Vol. 102.)

by these anaerobes. His keen intuition and observation were not susceptible of quantitative confirmation until the pioneer investigations of Clark¹⁰ on the oxidation-reduction equilibria of the indigo sulfonates. With 99 per cent reduction of the disulfonate, the potential of the system at pH 7 (30° C.) is, $E_h = -0.185$ v. From the value of the theoretical oxygen electrode (1.23 v.) one may calculate the pressure of O_2 at equilibrium with the dye system at -0.185 v. from the equation: $E_h = 1.23 - 0.06 \text{ pH} + 0.015 \log p_{O_2}$. The value

for the O_2 -pressure comes out $10^{-66.3}$ atm., equivalent to one molecule of oxygen in over 10^{43} liters. Note well that this conclusion states merely that there can be no appreciable O_2 in equilibrium with a reduced indigo disulfonate solution. It does not say that O_2 because of its inertness may not remain if originally present; however, this possibility seems exceedingly remote in active cultures of butyric anaerobes such as Pasteur had studied.

The indicator method of study (pursued by Paul Ehrlich many years before theory had sufficiently developed) was used in conjunction with the electrode by Clark and Cohen,^{11,12} who found that suspensions or cultures of various cells produce but little material possessing electromotive activity; nevertheless the cells are able to produce such material slowly, yet definitely. In the absence of oxygen, the electrode potential tends to approach that of the hydrogen system and often falls into the region of hydrogen "overvoltage." In the presence of bubbling oxygen, the potential fluctuates in a region far removed from the theoretical oxygen electrode.

Cultures of strict and facultative anaerobic bacteria can produce very negative reduction potentials. For instance, *Cl. welchii* and *Esch. coli*¹² readily reduce a sugar medium to the zone of the hydrogen electrode potential; the reduction intensity approaches and then passes beyond the potential ($E_h = -0.42$, at pH 7) at which hydrogen at atmospheric pressure is in equilibrium with the medium, and indeed such cultures liberate hydrogen at this stage.

Explorations of the cell interior (limited to a few types of organisms) have amplified the above findings.¹³ The most nearly complete study yet made is that on *Ameba dubia* by Cohen, Chambers and Reznikoff¹⁴ and by Cohen and Chen¹⁵ by means of the micro-injection of indicators. It was found that the cytoplasm of an ameba under purified moist nitrogen reduces the indophenols and indamines instantly (see p. 522), the intermediate indicators rather rapidly, and some of the more negative ones more slowly but yet completely. Finally, a level is reached where safranin T ($E'_0 = -0.289$, pH 7) remains partly reduced, and sulfonated rosindone ($E'_0 = -0.38$, pH 7) remains unreduced. The last two indicators, when injected in the reduced state, reoxidize at once, more or less completely in the cell interior. This fixes a limiting zone of oxidation-reduction potential under anaerobiosis for the ameba under the experimental conditions.

An entirely different situation obtains in the ameba exposed to moist air; for the intracellular reduction of the indicators extends only to ethyl Capri blue which remains about half reduced ($E'_0 = -0.072$, pH 7), corresponding to an equilibrium oxygen pressure of 10^{-59} atm. Such gas pressures are calculation values of no physical reality; but their significance is apparent.

The same general tendencies seem to obtain for other large cells.

The behavior of oxygen towards the living cell is not unique, for oxygen is notoriously inert and requires the intervention of a catalyst

even in purely inorganic reactions; *e. g.*, the reaction $\text{H}_2 + \frac{1}{2}\text{O}_2$ which is thermodynamically possible ($\Delta F^\circ_{298} = -56,500$ calories) does not occur except with a catalyst, such as palladium, when explosive oxidation results. The biologically important thing is the peculiar chemical mechanism of the cell whereby oxygen is activated at a rate and to a level adapted to the needs for optimal maintenance. The problem may be defined as follows: by what mechanism of catalysis is the theoretical potential of atmospheric oxygen ($E'_0 = +0.81\text{v.}$, pH 7) adapted to the low level observable in the aerobic cell (-0.07 volt)? It may be stated at once that the factor of physical diffusion of oxygen is of secondary importance. In this connection, it must be realized that the net, overall potentials observable in the cell cytoplasm do not preclude the existence of not readily detectable localized regions having markedly different reduction potentials.

Until the cellular reduction potentials are related to definite equilibria of fixed or transient systems, the actual physical measurements cannot be given the ordinary interpretation; and one has a right to question the nature of the quasi-equilibria shown by the electrode or indicators, and their fundamental significance. Clark¹⁸ states: "To the extent that these actual physical measurements may be related to definite biological events, they command respect as having met *biological* criteria of significance." Support for this is found in many observations, only several of which can be mentioned. Iodine, an oxidizing disinfectant, may be fed in many times the dose necessary to sterilize a culture medium without observable destructive effect, provided that it is added at such a rate that the potential in the medium does not rise appreciably above the normal level for the culture.¹⁸ Germination of tetanus spores¹⁹ and initiation of bacterial growth²⁰ have been related to the reduction potential of the environment. In addition, we may cite one of many *biochemical* events which shows a close correlation with the intracellular potential. The well-known ability of metabolites to reduce methylene blue (as in the Thunberg technic) finds its reflection in the ability of the aerobic cell to maintain a potential at which this dye can be completely reduced. Other correlations will become apparent in the paragraphs to follow.

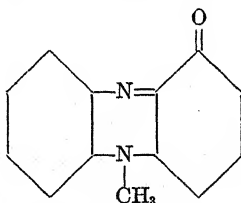
The examination of the cell as a whole under controlled conditions is of importance in getting the "lay of the land." Study of isolated biochemical systems will furnish the materials for constructing a solid path of understanding. Both approaches are necessary for progress.

POTENTIALS IN ISOLATED BIOCHEMICAL SYSTEMS

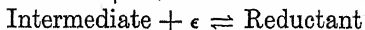
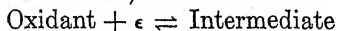
It is important to recognize that all oxidation-reduction systems do not have that labile thermodynamic reversibility necessary to yield equilibrium data. In some cases, practically instant equilibrium is attained and maintained; in others, this is followed by rapid decomposition of an unstable component; in still others, equilibrium can be attained only when a suitable catalyst is present alone or together with

another, electromotively active mediating system. By "electromotively active" is meant the ability of a system to affect an "inert," noble metal electrode as it is affected in a reversible galvanic cell. This calls attention to the very important and complex problem of phenomena at the electrode surface which remains an interesting field for investigation.

Natural Pigments.—A number of natural pigments have been found to be components of reversible oxidation-reduction systems, some of them capable of functioning as "respiratory catalysts"²⁷ (also see below). *Hermidine* occurs in the green shoots of *Mercurialis perennis* and, according to Cannan,²⁸ it is at least 95 per cent reduced within the cells while adjacent plastids are producing molecular oxygen. The E'_0 of this pigment at pH 7 is approximately -0.03 v., which would give for 95 per cent reduction a potential of -0.068 v. Compare this with -0.07 v. found in the aerobic ameba. *Echinochrome*, present in many Echinoderms in the oxidized state was found to have an E'_0 of -0.228 v. at pH 7.²⁸ *Chromodoris zebra* pigment, apparently relatively little reduced in the cells, has an E'_0 of -0.102 v. at pH 7.²⁹ *Pyocyanine* from *B. pyocyaneus* occurs in the reduced form within the bacterial cell, and has an E'_0 of -0.034 at pH 7.³⁰ Of interesting theoretical significance is the fact that this compound:



represents a group of organic systems in which the usual reduction: Oxidant + $2e \rightleftharpoons$ reductant, involving two, *paired*, equivalents at one region of pH becomes, at another pH, separated into two distinct stages involving one equivalent each, thus:



Michaelis³¹ considers that the intermediate compound is of the nature of a free radical and suggests that such free radicals or semiquinones may occur more widely in living organisms than has been hitherto suspected.

Lactoflavine ($C_{17}H_{20}N_4O_6$) occurring both free and as a component of flavoprotein, Warburg's³⁶ yellow "respiratory" pigment, has an E'_0 of -0.21 at pH 7.³⁷ Lactoflavine has also been identified as related to vitamin B₂.*

Methemoglobin.—The system: Hemoglobin + $O_2 \rightleftharpoons HbO_2$ is not electromotively active, and Conant³² distinguishes this as an *oxy-generation* quite distinct from an oxidation in the usual sense. On the other hand, in the oxidation to methemoglobin, the system: $Hb \rightleftharpoons \text{MetHb} + e$ is electromotively active, involving the transformation

* See p. 507.

of the ferrous iron in hemoglobin to the ferric in methemoglobin. The position of this system on the E'_0 : pH diagram is shown as the dotted curve L on page 525. Considerable technical difficulties stand in the way of obtaining exact reproducibility of potentials. Interesting observations are also reported on the copper-containing respiratory pigment *hemocyanin*³³ for it appears that both the oxyhemocyanin and the methemocyanin systems are electromotively active. Measurements of *hemins* have been reported by Conant, Alles and Tongberg³⁴ and of *cytochrome C* by Green.³⁵ These observations are of significance because special hemochromogens are now more and more becoming associated with oxidase, peroxidase and catalase activities (see below); and their electromotive behavior reveals them as more or less reversible oxidation-reduction systems.

Reversible Oxidation-reduction Mediators.—The reaction: $\text{COOH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} \rightleftharpoons \text{COOH} \cdot \text{CH} = \text{CH} \cdot \text{COOH} + 2\text{H}^+ + 2e$ does not proceed with measurable velocity. Addition of a suitable enzyme tends to speed it up but electromotive equilibrium is not attainable. The introduction of a small amount of a reversible system lying in the same region of potential promotes prompt attainment of equilibrium which can be measured by means of an indicator or an electrode.³⁹ Investigation of this important mechanism shows that the process satisfies the necessary criteria of thermodynamic reversibility⁴⁰ and that the enzyme functions as a true catalyst. When methylene blue is the added reversible system (mediator), the reaction in the presence of enzyme proceeds reversibly thus: Succinate + methylene blue \rightleftharpoons fumarate + leuco-methylene blue; and at 45° C. and pH 7.2 the value of the equilibrium constant is 3 when the ratio of methylene blue to its leuco-product equals one. That is,

$$\frac{(\text{Succinate}) (\text{Methylene blue})}{(\text{Fumarate}) (\text{Leuco-methylene blue})} = 3.0$$

From the data for the dye system,⁶ after proper corrections, one can determine the electrode potential of the succinate-enzyme-fumarate system. It should be emphasized that the reversible dye system need be present only in low ratio (one thirtieth or less) to the principal reactants and that it acts as a catalytic go-between which does not significantly alter the main equilibrium. Borsook and Schott⁴⁰ have shown that the standard free energy change for the above reaction is: $\Delta F^\circ_{298.1} = -20,180$ calories as determined by the indicator method, $-20,140$ calories as determined by the electrode method, and $-20,460$ calories as calculated from the entropies and other energy properties of succinic and fumaric acids. At 30° C., E'_0 for the system is about -0.02 v. at pH 7.

This phenomenon (action of reversible mediators) is of great significance. In the first place, it furnishes a means for determining the free energies of some systems in which the electrode alone is useless. Secondly, the ability of reversible systems in general to act as medi-

ators in oxidation-reduction reactions is undoubtedly utilized in cellular processes. The essential condition for an indicator to function as a mediator in establishing equilibrium of a sluggish reaction such as the above is that the indicator shall be reversible and electromotively active so that its oxidant may react spontaneously with the sluggish reductant and its reductant with the sluggish oxidant.

The same principle has been applied to the measurement of the lactate-enzyme-pyruvate system.⁴¹ E'_0 at 37° C. is approximately -0.16 volt at pH 6.7.

Measurements of epinephrine (adrenaline) and related compounds have been made³⁸ and the E'_0 : pH curves are shown in Fig. 37. Epinephrine in the natural state occurs as the reductant, and it has been shown that the oxidant in neutral solution (pH 7.66, $E'_0 = +0.345$) is very unstable, having a half-life of 0.06 second. The potential is so positive that living tissues can keep the system in the reduced state, thus protecting the epinephrine from destructive oxidation.

Wurmser and Geloso find in sugar solutions indications of transitory changes with relatively slow approach to rather characteristic potentials comparable to those found in biological systems.

CHEMICAL MECHANISMS*

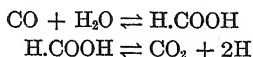
For an account of the various chemical processes occurring in biological oxidations and reductions reference should be made to the monograph by Dakin.⁴² Only some of the more general features can be touched upon here. Every oxidation implies a reciprocal reduction, and the process involves primarily an activation of either or of both components, since relatively few such reactions proceed spontaneously with any speed. Since the process may occur through a transfer of electrons, of hydrogen or of oxygen, alone or in combination, a loose or exclusive emphasis on any one mechanism may lead to error.

Dehydrogenation.⁴³—Many of the organic compounds oxidized in the body are quite stable towards oxygen. Some of them, *e. g.*, succinic acid, can withstand boiling in strong nitric acid. Yet in the presence of appropriate cell catalysts, they become readily oxidized at body temperature through a loss of hydrogen. The reaction (in the presence of catalyst) may be written: $AH_2 + B \rightleftharpoons A + BH_2$; a typical example is the succinate-enzyme-fumarate reaction with methylene blue. The biological catalysts which activate the hydrogen of the substrate are of the class of *dehydrogenases*. The mechanism of the activation of hydrogen in organic compounds probably involves a shift of electrons with weakening of certain valence bonds.⁴⁴ In this connection may be mentioned the hypothesis of Haber and Willstätter⁴⁵ that, in enzymatic oxidation-reduction, the enzyme is univalently reduced while the substrate is univalently oxidized. The latter product is of the nature of a free radical which can link either to oxygen or more readily to carbon. Continual repetition of this univalent oxi-

*See p. 473.

dation-reduction is imagined to give reaction chains leading to the end-products.

It has been found that the oxidation of CO can proceed through the following steps:



The essential change in this reaction involves the removal of hydrogen from an unstable intermediate addition compound; and oxidation, strictly speaking, begins with the removal of the hydrogen, no free oxygen being required. Water, however, is necessary in this process, which is typical of an important group of biological oxidations. It will be noted that the second step is an intramolecular oxidation-reduction. According to the mass law, the process can proceed to completion if one of the end-products is removed; *i. e.*, hydrogen taken up by a hydrogen "acceptor," or CO_2 by some CO_2 - "acceptor." The emphasis in the literature has been placed upon hydrogen mobilization (and activation) and hydrogen acceptors. This is legitimate where actual hydrogen transfer is involved, but the meaning of the concept becomes indefinite in those cases where the calculated equilibrium pressures of hydrogen are of the order of 10^{-20} atm., as it is with half-reduced indophenol in washed tissue suspension at pH 7.²⁰ The generalization of this concept certainly is false when applied to cytochrome as in the following statement "oxygen combines with the hydrogen of [reduced] cytochrome."

Peroxides.—A number of organic compounds when undergoing slow oxidation yield reactions for peroxide (Dakin). In the transformation of HCOOH to CO_2 and 2H , the hydrogen can react with O_2 to give H_2O_2 . Benzaldehyde can add a molecule of oxygen to form benzoyl hydrogen peroxide, a powerful oxidizing agent. It seems quite likely that unstable peroxides are concerned with biological oxidations. Dakin points out the extraordinary similarity of many types of reactions brought about by H_2O_2 *in vitro* and certain specific oxidations in the body. For example, H_2O_2 alone of all the various chemical oxidizing agents brings about precisely the same conversion of butyric acid to acetoacetic acid. It would seem, however, that in the body it is an organic peroxide rather than H_2O_2 which is the active agent.

Cannizzaro Reaction.—The intermolecular oxidation-reduction known by this name is a characteristic reaction of aldehydes whereby two molecules interact with water to give the corresponding acid and alcohol:



This process is accelerated by a tissue catalyst named *aldehydemutase*. In general, it may be said that aldehydes may undergo this rearrangement in the body.

Activation of Oxygen.—Molecular oxygen without a catalyst apparently cannot participate in many biological oxidations. The possibility of peroxide formation has already been mentioned. The

accelerating effect on oxidations by peroxides exerted by salts of iron and manganese were long ago observed. It is due to the researches of Warburg⁴⁶ that we have a clearer appreciation of the rôle of iron in biological oxidations. Evidence is now accumulating that the iron-containing material in cells which activates oxygen belongs to the class of hemochromogens. This material acts like a hematin bound to a nitrogenous substance forming a highly catalytic and autoxidizable compound which can rapidly undergo oxidation-reduction.⁴⁷ It has apparently the same properties and distribution as indophenol-oxidase. We have already mentioned the reversible oxidation-reductions of ferrous to ferric hemochromogens. It seems that the indophenol-oxidase is of the type of the Warburg iron catalyst (oxygen activator) in the sense that it becomes oxidized from the ferrous to the ferric state. It is interesting to note that of the few such oxidases thus far discovered, indophenol-oxidase alone has a very wide distribution in nature, can be inhibited by KCN and CO, reactivated by light after CO-poisoning and can oxidize reduced cytochrome.

Cytochrome^{47*} is a complex of hematins combined with nitrogenous substances and of wide distribution in nature in aerobic cells. It is absent from anaerobic cells. There is a certain parallelism between the concentration of this pigment and the respiratory activity in cells. Reduced cytochrome is not oxidizable by atmospheric oxygen. In the autoxidation of cysteine, indophenol-oxidase or cytochrome alone cause a very small oxygen uptake whereas the oxidase plus cytochrome cause a very high oxygen uptake. It would seem that this is a coupled process whereby the oxygen is activated to the extent of converting the catalyst system (oxidase) to its oxidized component, followed by a second reversible step through the cytochrome, somewhat like high tension commercial current is stepped down to the needs of motors, etc., in a factory.

Reversible Systems Acting Directly with Oxygen.—It is well known that reduced methylene blue and the reductants of many other reversible dye systems will reoxidize on exposure to air. The reaction occurs as if directly between dye reductant and oxygen. To this class of substances belongs the yellow fluorescent "respiratory" pigment (flavoprotein) found by Warburg and Christian³⁶ as a widely distributed cell component, and distinct from the homochromogen system.†

Peroxidase.⁴⁸—The catalytic activity of iron and manganese in oxidations by peroxide has been mentioned. This *peroxidase* activity is found to be possessed in very strong degree by cytochrome and other hematin compounds which are responsible for the peroxidase reaction in yeasts, bacteria and hemoglobin-free tissues of animals (Keilin). A highly purified preparation of peroxidase showed approximate proportionality between hemochromogen content and activity.

Catalase, the cell catalyst which accelerates the conversion of H_2O_2 to water and molecular oxygen has also been found to be related to the hemochromogens⁴⁹ with an absorption spectrum similar to that

* See p. 499. † See p. 499.

of methemoglobin. The biological significance of catalase has not been established. It is assumed to protect the cells from an overaccumulation of H_2O_2 .

Glutathione and Sulfhydryl Compounds.—The presence of reduced sulfur-containing compounds in living tissue is well known. That they probably constitute a factor in tissue oxidations became clear when O. Meyerhof observed the respiratory activity of SH groups and Thunberg³⁹ the autoxidizability of cysteine. Of chief importance, because it is apparently present in all cells is glutathione,⁵⁰ the tripeptide glutamyl-cysteinyl-glycine. It is readily oxidizable *in vitro* by molecular oxygen to the disulfide form provided iron or copper is present. The disulfide is readily reduced by tissues and seems to promote the oxidation of unsaturated fats and amino acids. Reduced glutathione is able to decolorize methylene blue but attempts to determine the potential of any sulfhydryl: disulfide system have met with peculiar technical difficulties in that the electrode seems ordinarily to respond only to changes in concentration of the reductant.⁵¹ It appears, however, that suitably protected electrodes can detect significant (equilibrium) potentials.⁵² Perhaps of greatest significance is the ability of the thiol: disulfide systems to form complexes with metal ions which probably endows them with their peculiar properties in general and specific cellular oxidations.

Relation to Hydrolytic Enzymes.—An important recent development has been the discovery that the oxidation-reduction state may be a controlling factor in the kinetics of certain enzymatic processes.⁵³ Certain hydrolytic enzymes have been found to become inactivated upon controlled treatment with mild oxidizing agents and restored to activity on reduction. An instructive example is shown in the following table from the data of Hellerman.*

REVERSIBLE INACTIVATION OF HYDROLYTIC ENZYMES

| Urease. | Relative activity. | Papain. | Relative activity. |
|------------------------------|--------------------|------------------------------|--------------------|
| Control | 100 | Control | 100 |
| " + iodine | 0 | " + iodine | 0 |
| " + " + H_2S | 40 | " + " + H_2S | 100 |

Other oxidizing agents besides iodine may be used for inactivation; and other reducing agents besides H_2S for reactivation. There are strong indications that sulfhydryl groups associated with the enzyme itself, at least for papain, are involved in this reversible inactivation. In addition, we are told that amylolysis is activated by disulfide glutathione⁵⁴ while proteolysis is activated by sulfhydryl. The whole field presents many interesting complexities, which, when solved, should go far toward elucidating the degree of control on hydrolytic reactions exercised by the oxidation-reduction environment.

* Personal communication.

Conclusion.—The student of biochemistry must always keep in mind the physical configuration of the living cell if he is to analyze and appreciate intelligently the relations of the more or less isolated phenomena just presented to that integrated process called cell respiration. The cellular material is not a homogeneous solution, but a complex of innumerable phases and of structures within structures. There is much evidence pointing to important chemical reactions taking place at interphase boundaries, surfaces which may well aggregate a square meter in area for a single microscopical cell.

It is conceivable that certain of the biochemically important components of a reaction are segregated within restricted regions of the cell.⁴⁴ In such a case, the purely physical mechanism whereby the necessary participants in a reaction are brought together in this heterogeneous, polyphasic unit may be the significant factor governing the *rates* of biological oxidations and reductions.

Our discussion of the subject has dealt with isolated systems of different sorts which are more or less definitely identifiable. It is important, in this connection, to realize that in the living cell a "half-reaction" does not take place, any more than it does in an electrical half-cell. In the latter, for instance, the change $\text{Fe}^{+++} \rightleftharpoons \text{Fe}^{++}$ can take place only if this half-cell is coupled to another suitable half-cell; *e. g.*, $\text{H} : \text{H}^+$, in such a manner that the conversion in the first process is made at the expense of the second. It is, of course, apparent that when the configuration of the electrical cell is removed there is no actual electrode potential, but nevertheless, the intensity or potential of the chemical system involved remains to make itself manifest (as heat, light, electricity, chemical energy) whenever the appropriate path is provided. In the living cell, there seems to be no mechanism corresponding to metallic conduction of electrons. Therefore the transport of energy from the point where it is liberated to the point where it is absorbed must take place through chemical energy carriers or mediators with functions like those demonstrated in the reversible oxidation-reduction indicators. By defining the mechanism in these terms we are not merely substituting a newer name, "mediator" for another, older name, "respiratory substance," but assigning a definite function, experimentally demonstrable, which logically integrates an important phase of our knowledge of biological oxidations. Coupled reactions⁵⁵ no doubt occur within living cells; and one of the problems of the immediate future is the discovery and examination of the naturally occurring mediators within the cells. Many of the cell pigments mentioned in the preceding pages have this property; but there is no apparent reason why it should be confined to substances with a spectral absorption in the visible region.

Finally, we depend in a very vital way on the chemical examination of cell metabolites and the identification of the various steps in their degradations and syntheses for the facts wherewith to interpret and reconstruct the mechanisms and kinetics of biological oxidations.⁵⁶

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REFERENCES

1. Lewis, G. N., and Randall, M.: *Thermodynamics* (1923).
2. Macdougall, F. H.: *Thermodynamics and Chemistry* (1926).
3. Baas-Becking, L. G. M., and Parks, G. S.: *Physiol. Rev.*, **7**, 89 (1927).
4. Burk, D.: *J. Phys. Chem.*, **35**, 432 (1931).
5. Wilson, P. W., and Peterson, W. H.: *Chem. Rev.*, **8**, 427 (1931).
6. Clark, W. M., Cohen, B., et al.: *Hygienic Laboratory Bulletin*, No. 151 (1928).
7. Wurmser, R.: *Oxydations et Réductions* (1930).
8. Michaelis, L.: *Oxydations-Reductions-Potentiale* (1933).
9. Ball, E. G., and Chen, T.-T.: *J. Biol. Chem.*, **102**, 691 (1933).
10. Clark, W. M.: *J. Wash. Acad. Sci.*, **10**, 255 (1920); **14**, 123 (1924).
11. Clark, W. M., and Cohen, B.: *Abstracts Bact.*, **6**, 3 (1922).
12. Cohen, B.: *J. Bact.*, **15**, 16 (1928); **21**, 18 (1931).
13. Chambers, R.: *Cold Spring Harbor Symposia*, **1**, 205 (1933).
14. Cohen, B., Chambers, R., and Reznikoff: *J. Gen. Physiol.*, **11**, 585 (1928).
15. Cohen, B., and Chen, T.-T.: *Proc. Soc. Exptl. Biol. Med.*, **21**, 115 (1933).
16. Cohen, B.: *Cold Spring Harbor Symposia*, **1**, 195, 214 (1933).
17. Michaelis, L., and Hill, E. S.: *J. Gen. Physiol.*, **16**, 859 (1933).
18. Clark, W. M.: *Harvey Lectures* (1933).
19. Fildes, P.: *Brit. J. Exp. Path.*, **10**, 151 (1929).
20. Allyn, W. P., and Baldwin, I. L.: *J. Bact.*, **23**, 369 (1932).
21. Hall, W. L., Preisler, P. W., and Cohen, B.: *Public Health Repts.*, Supplement No. 71 (1928).
22. Cohen, B., and Phillips, M.: *Public Health Repts.*, Suppl. No. 74 (1929).
23. Phillips, M., Clark, W. M., and Cohen, B.: *Public Health Repts.*, Supplement No. 61 (1927).
24. Cohen, B., and Preisler, P. W.: *Public Health Repts.*, Suppl. No. 92 (1931).
25. Stiehler, R. D., Chen, T.-T., and Clark, W. M.: *J. Am. Chem. Soc.*, **55**, 891 (1933).
26. Stiehler, R. D., and Clark, W. M.: *J. Am. Chem. Soc.*, **55**, 4097 (1933).
27. Friedheim, E. A. H.: *Compt. rend. soc. biol.*, **112**, 1030 (1933).
28. Cannan, R. K.: *Biochem. J.*, **20**, 927 (1926); **21**, 184 (1927).
29. Preisler, P. W.: *J. Gen. Physiol.*, **13**, 349 (1930).
30. Friedheim, E. A. H., and Michaelis, L.: *J. Biol. Chem.*, **91**, 355 (1931).
31. Michaelis, L.: *Cold Spring Harbor Symposia*, **1**, 224 (1933).
32. Conant, J. B., and Pappenheimer, A. M.: *J. Biol. Chem.*, **98**, 57 (1932).
33. Conant, J. B., Chow, B. F., and Schoenbach, E. B.: *J. Biol. Chem.*, **101**, 463 (1933).
34. Conant, J. B., Alles, G. A., and Tongberg, C. O.: *J. Biol. Chem.*, **79**, 89 (1928).
35. Green, D. E.: *Proc. Roy. Soc. (London)*, **114B**, 423 (1934).
36. Warburg, O., and Christian, W.: *Biochem. Z.*, **254**, 438 (1932); **258**, 496 (1933).
37. Bierich, R., and Lang, A.: *Z. physiol. Chem.*, **223**, 180 (1934).
38. Ball, E. G., and Clark, W. M.: *Proc. Natl. Acad. Sci.*, **17**, 347 (1931).
39. Thunberg, T.: *Skand. Arch. Physiol.*, **40**, 1 (1920); **46**, 339 (1925).
40. Borsook, H., and Schott, H. F.: *J. Biol. Chem.*, **92**, 535 (1931).
41. Barron, E. S. G., and Hastings, A. B.: *J. Biol. Chem.*, **100**, 155 (1933).
42. Dakin, H. D.: *Oxidations and Reductions in the Animal Body* (1922).
43. Wieland, H.: *Ber.*, **45**, 484, 679, 2606 (1912); **46**, 3327 (1913); **47**, 2085 (1914).
44. Quastel, J. H.: *Biochem. J.*, **20**, 166 (1926).
45. Haber, F., and Willstätter, R.: *Ber.*, **64B**, 2844 (1931).
46. Warburg, O.: *Ueber die katalytischen Wirkungen der lebendigen Substanz* (1912-1928).
47. Keilin, D.: *Ergebnisse Enzymforsch.*, **2**, 239 (1933).
48. Hand, D. B.: *Ergebnisse Enzymforsch.*, **2**, 272 (1933).
49. Zeile, K., and Hellström, H.: *Z. physiol. Chem.*, **192**, 171 (1930).
50. Hopkins, F. G.: *J. Biol. Chem.*, **84**, 269 (1929).
51. Michaelis, L., and Flexner, L. B.: *J. Biol. Chem.*, **79**, 689 (1928).
52. Ghosh, J. C., and Ganguli, S. C.: *Biochem. J.*, **28**, 381 (1934).
53. Hellerman, L., Perkins, M. E., and Clark, W. M.: *Proc. Natl. Ac. Sci.*, **19**, 855 (1933).
54. Pringsheim, H., Borchardt, H., and Hupfer, H.: *Naturwissenschaften*, **20**, 64 (1932).
55. Schott, H. F., and Borsook, H.: *Science*, **77**, 589 (1933).
56. Meyerhof, O.: *Nature*, **132**, 337, 373 (1933).

CHAPTER XX

CARBOHYDRATE METABOLISM

MANY of the brilliant observations and conceptions of the French physiologist, Claude Bernard, are still the foundation of our knowledge of carbohydrate metabolism. Between 1849 and 1857 he showed that the sugar in the blood is glucose, that animal tissues contain a peculiar starch, glycogen, and that nervous centers enter into the control of the glucose concentration in the blood. In 1886 Minkowski in Germany observed that hepatectomy was followed by a disappearance of sugar from the blood. Thus it was shown that the liver is concerned in the production of blood sugar. This finding was confirmed and greatly amplified by Mann and his coworkers in this country (1924). Mering and Minkowski made the momentous discovery that extirpation of the pancreas from dogs makes these animals diabetic. In their paper, published in 1890, they gave such a complete description of the symptoms of the disease that little could be added later.

The glycosuric effect of epinephrine, the hormone of the adrenal medulla, was discovered by Blum in 1902. In 1922 the Canadians, Banting and Best, succeeded in the extraction of a hormone from the pancreas which they called *insulin*. This important achievement has enlarged our knowledge and understanding of carbohydrate metabolism, besides saving the lives of many diabetic sufferers. The importance of yet another endocrine gland for carbohydrate metabolism was emphasized in 1929 by the Argentinean, Houssay, who found that in hypophysectomized animals, pancreatectomy failed to produce the usual severe symptoms of diabetes. The Englishmen, Harden and Young, discovered the formation of hexose phosphoric acid esters in yeast; esterification with phosphoric acid was later shown to be the first step in the anaerobic breakdown of carbohydrate in yeast and animal tissues.

Many of the discoveries just enumerated were made possible by improvements in chemical methods. Claude Bernard and his contemporaries had to use large amounts of blood for sugar determination, a fact which was obviously a limiting factor for many lines of physiologic experimentation. Gradually, chemists succeeded in devising methods for the determination of small quantities of glucose. From beginnings such as the determination of sugar in 25 cc. of blood by Fehling's titration, micromethods were developed which permit the accurate determination of sugar in 0.1 cc. of blood. The Scandinavian, Bang, and Folin, S. R. Benedict, Shaffer and Somogyi in this country are mostly responsible for this achievement. An accurate determination of glycogen in tissues was only possible after the German physiologist, Pflüger, devised his remarkable method in 1901, which in its

essential features is still the method of choice today. The Austrian, Fürth, introduced the principles of lactic acid determination, which in the hands of Shaffer and his coworkers were developed into a method for the accurate and speedy determination of minute quantities of lactic acid in blood and tissues.

Intestinal Absorption.—Carbohydrates constitute a large, sometimes the major portion of food consumed by herbivorous and omnivorous animals. The carbohydrates occurring in food are generally complex in nature, such as cellulose, starch, glycogen, inulin, pentosans, mannans, to name only a few. The complex carbohydrates cannot be absorbed as such from the gastro-intestinal tract. In order to make them available to the organism in an absorbable form, they have to be acted upon by digestive enzymes, which break them down to smaller complexes and finally to monosaccharides. Cellulose is not readily attacked by the digestive enzymes of man and thus fails to be assimilated to any appreciable extent; it passes the gastro-intestinal tract largely unchanged and provides the necessary roughage of our diet. Ruminants and other herbivorous animals are capable of utilizing a considerable quantity of cellulose and they are aided in this by a special bacterial flora. These bacteria carry out the first step of hydrolysis of cellulose, which is then completed by the digestive enzymes.

Even the common disaccharides sucrose, lactose and maltose, have to undergo hydrolysis into their constituent monosaccharides before an appreciable absorption can take place. Glucose, fructose and galactose are the most important end-products of enzymatic hydrolysis of carbohydrates in the digestive tract. In addition, mannose and various pentoses may be formed.

Absorption of monosaccharides takes place in the small intestine and follows rather definite laws which were established in experiments on rats and dogs following the administration of solutions of various sugars.¹ Cognizance of the various factors governing absorption is of importance for the interpretation of glucose tolerance tests.

Different monosaccharides are not absorbed at the same rate. Glucose, galactose and fructose are absorbed more rapidly than mannose and pentoses. The difference in the rate of absorption of various sugars may be explained by selective action of the intestinal mucosa. The following order in the rate of absorption was established in experiments on rats and dogs: Galactose > glucose > fructose > mannose > xylose > arabinose. When mixtures of two sugars or of sugars and amino acids are fed, the rate of absorption of each component of the mixture is reduced. Similarly, after a mixed meal, owing to the presence of different digestive products all undergoing absorption at the same time, the rate of absorption of sugar is considerably slower than when the sugar is taken in pure solution. In addition to the selective action of the intestinal mucosa and the effect of the presence of other substances undergoing absorption, the rate of absorption of sugar

is limited by the rate at which polysaccharides and disaccharides are split into monosaccharides by the digestive enzymes. It is for this reason that starch produces a smaller rise in blood sugar than an equivalent quantity of glucose, and the same applies to lactose as compared to a mixture of equal parts of glucose and galactose.

The rate of absorption of glucose and other monosaccharides remains fairly constant until most of the sugar is absorbed. The amount of the sugar fed merely determines the length of time of absorption but not the rate of absorption. The following figures taken from a recent paper² illustrate this point. A dog of 17 kilos received 26.9 Gm. of glucose in 4.5 per cent solution and absorbed the sugar at a rate of 0.84 Gm. per kilo per hour, while another dog of similar weight after receiving 45.5 Gm. in 46.4 per cent solution absorbed glucose at a rate of 0.8 Gm. per kilo per hour. Even though the quantity of glucose was nearly doubled and the concentration increased tenfold, the rate of absorption remained practically the same. The only difference was that in the second case complete absorption of the sugar fed took twice as long as in the first case.

How can such a mechanism of absorption be explained? Recently the interesting suggestion has been made that esterification with phosphoric acid plays an important rôle in the absorption of glucose.^{3, 4} If this should prove to be the case, it may explain why only a certain number of glucose molecules can pass through the intestinal epithelium per unit of time, irrespective of the amount and the concentration of sugar fed. The possibility that absorption of glucose may be dependent on phosphorylation is strengthened by certain facts known regarding phlorhizin, a glucoside, which prevents the reabsorption of glucose in the tubules of the kidney and thus leads to glycosuria. Phlorhizin also inhibits intestinal absorption of glucose⁴ and when added to tissues *in vitro* it has been shown to prevent esterification of glucose with phosphoric acid. The inference is that it diminishes intestinal absorption of sugar and reabsorption of sugar in the tubules of the kidney by inhibiting phosphorylation.

An interesting correlation exists between the rate of intestinal absorption and the capacity of different species to dispose of injected glucose.¹ Rats absorb glucose at a rate of about 2.2 Gm. per kilo per hour without excreting any sugar in the urine; and this also happens to be the maximum rate at which glucose may be injected intravenously in rats without causing glycosuria. In dogs the rate of absorption of glucose is slower, namely, 0.9 Gm. per kilo per hour;² and the intravenous tolerance rate is correspondingly lower, namely, 0.9 Gm. per kilo per hour.⁵

Only insignificant amounts of glucose are absorbed in the stomach. When a known amount of sugar is introduced and the pylorus occluded, 99 per cent can be recovered several hours later.⁶ Yet the stomach may indirectly influence absorption. Ordinarily, sugar is always present in the small intestine while absorption is in progress,

indicating that the stomach delivers the sugar solution at a rate faster than it is absorbed in the small intestine. Under certain pathologic conditions, and after the injection of drugs such as atropine, morphine and amytal, the passage of sugar from the stomach to the intestine is delayed so that, at times, its concentration in the small intestine may fall to zero. The result is a reduced and irregular rate of absorption. During inanition and vitamin B₁ deficiency the rate of absorption of glucose is diminished, but it has not been ascertained whether or not a stomach factor is involved.

After ingestion of glucose, the lower ileum generally contains very little sugar, so that the large intestine does not ordinarily participate in the absorption of sugar. However, the large intestine is capable of absorbing sugar, as shown in experiments with rectal administration of glucose and fructose.⁷

During absorption, blood flow through the intestine is increased very markedly. Since most of the sugar which has passed the intestinal wall is carried away by the blood stream, circulation is an important factor in the absorption process.

Blood and Tissue Sugar.—The sugar circulating in the blood under postabsorptive conditions is mostly α -, β -glucose, as shown by a comparison of polarimetric and copper reduction values obtained on suitably prepared blood filtrates.⁸ In man and monkeys glucose is present both in the corpuscles and the plasma, and the ratio of corpuscle to plasma sugar is found to be about 0.8. On the basis of the water content of corpuscles and plasma, this represents a nearly equal distribution of sugar. The corpuscles of other species contain only small amounts and, in some cases, no sugar, so that the plasma sugar concentration is considerably higher than the sugar concentration in whole blood.⁹

The corpuscles contain a number of nonsugar substances, notably glutathione and thioneine, which have reducing properties. Estimations made with older methods gave blood sugar values which were too high, owing to the presence of these nonsugar reducing substances in the blood filtrates. Recently, several methods have been developed which permit the determination of the fermentable or "true" sugar content of the blood. It should be pointed out, however, that the nonsugar reducing substances remain fairly constant, so that the values obtained with older methods, while not representing "true" sugar, indicated quite accurately changes in blood sugar concentration.

The fermentable sugar content of human blood obtained twelve hours after the last meal is about 80 mg. per 100 cc. Other mammals show similar blood sugar concentrations, although in ruminants the concentration is generally lower than in man. Birds show a higher blood sugar concentration than mammals. The concentration in cold-blooded animals is very low; in the frog, for instance, values of 20 mg. per cent are commonly found. The life of a mammal could not be maintained at such a blood sugar concentration.

Apart from blood, all tissues contain fermentable sugar. In most tissues the sugar concentration is lower than in the blood. At a plasma sugar concentration of 100 mg. per cent, skeletal muscle contains only 10 to 15, and heart muscle 30 to 40 mg. per cent of fermentable sugar. The tissue sugar reflects changes in plasma sugar concentration. If the latter rises, there is a corresponding increase in the fermentable sugar of heart and skeletal muscle; and if the plasma sugar falls below the normal level, this is accompanied by a similar change in muscle sugar.¹⁰ Skin contains a larger amount of sugar than muscle.¹¹ Fermentable sugar is also found in the lymph, in the spinal fluid, in the aqueous humor of the eye and in transudates and exudates.

After ingestion of fructose, galactose, pentoses and of the triose dihydroxyacetone, their presence can generally be demonstrated in the blood. With the exception of pentoses, the liver is capable of converting the sugars mentioned to glucose either directly or after transformation to glycogen.

The sugar concentration of arterial blood (or of capillary blood, which is similar to arterial blood in composition) is generally higher than that of venous blood, owing to the fact that most tissues continually remove glucose from the blood stream. The difference between the arterial and venous blood sugar concentration of a limb has been found to increase after sugar ingestion and after insulin injection.¹² Administration of glucose plus insulin gives a greater difference than administration of glucose alone; this is illustrated in Fig. 38. In untreated diabetics with high blood sugar concentration the arterial-venous difference is generally very small. The observed difference between arterial and venous blood sugar concentration is only a qualitative index of the rate of sugar utilization in the tissues, unless the rate of blood flow through the limb is determined simultaneously.

Under physiologic conditions, the blood sugar concentration varies only within narrow limits. The blood sugar level depends on the relative rate at which sugar enters and leaves the blood stream. Maintenance of a constant blood sugar level requires an in- and outflow of sugar of equal magnitude. When there is an influx of sugar into the blood, as, for instance, during glucose absorption, provisions are made for a rapid removal of the sugar so that an undue rise in the blood sugar concentration is prevented. Four processes are known by which sugar can be disposed of: (a) Glycogen formation in liver, muscle and other tissues; (b) carbohydrate oxidation; (c) transformation of carbohydrate to fat; and (d) excretion of sugar in the urine. Each of these processes will be considered separately in the sections which follow.

In the fasting organism the sugar concentration in the hepatic vein is higher than in the portal vein and hepatic artery, indicating that the liver liberates sugar into the blood. After surgical removal of the liver in dogs, an operation which was perfected by Mann, the blood sugar concentration falls rapidly and the animal survives only

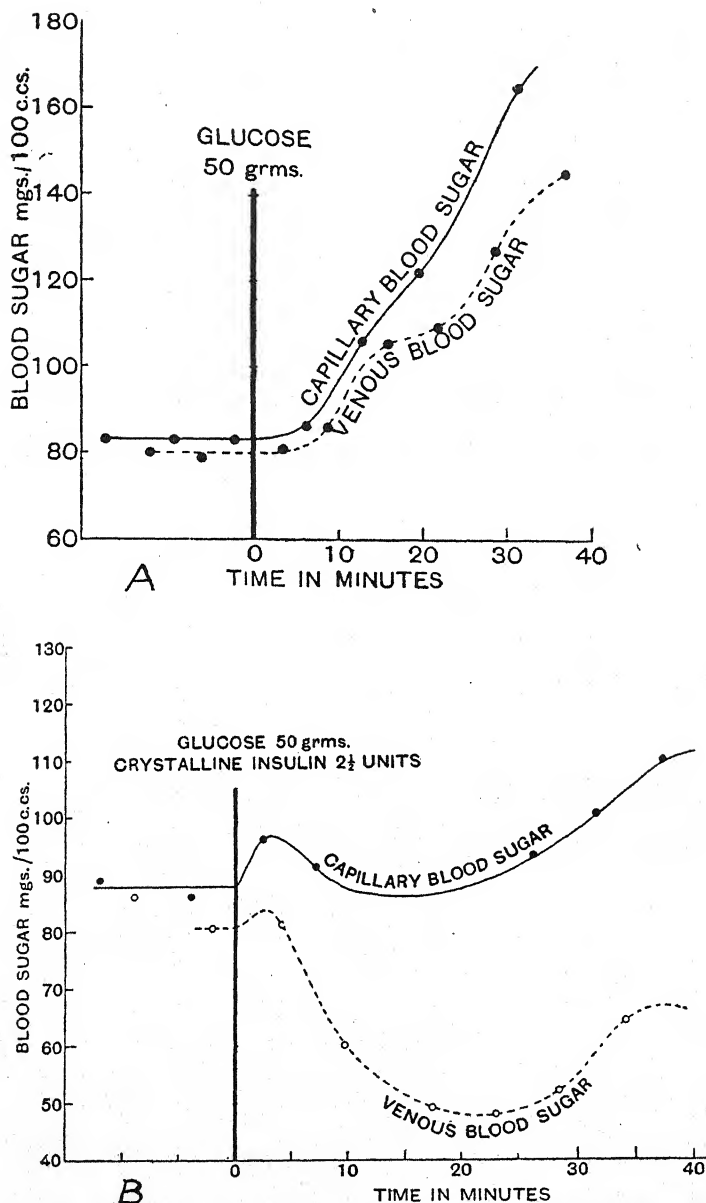


Fig. 38.—Capillary and venous blood sugar curve. Subject had been on a high fat diet for several days. *A*, After 50 Gm. of glucose. *B*, After 50 Gm. of glucose and 2.5 units of crystalline insulin. Subjects on a high fat diet show a greater rise in blood sugar after a glucose test meal than subjects on a high carbohydrate diet. Note the marked effect of insulin on the arterial-venous blood sugar difference. This is due to a more rapid utilization of blood sugar in the tissues under the influence of insulin. (Data from Himsworth.¹²)

a short time, unless sufficient glucose is injected to maintain a normal blood sugar concentration. Though the muscles of the hepatectomized animal contain an abundant supply of glycogen at the time that hypoglycemia develops, they do not contribute glucose to the blood. This experiment indicates that the liver is the chief and possibly the only source of blood sugar in the body.

When the organism is fasting, the blood sugar concentration is maintained by a continuous formation of sugar in the liver. This is of vital importance to the organism, because when the blood sugar falls below a certain level, very serious symptoms develop which may result in death from hypoglycemia. The sugar is formed by the liver from its glycogen. However, the glycogen content of the liver would soon become exhausted during fasting, if it were not for the fact that new glycogen is continuously being deposited in the liver. It is necessary, therefore, to consider, first, the sources of liver glycogen.

Glycogen Formation in Liver.—During absorption of glucose there is a considerable increase in the sugar concentration of the portal blood. The sugar is carried to the liver and may be retained there as glycogen, or it may pass the liver unchanged and find its way into the general circulation. In experiments on rats¹³ it was found that during four hours of absorption, about one out of every five molecules of glucose absorbed from the intestine was retained as liver glycogen.

The percentage of liver glycogen deposited from various sugars is shown in the following table. It may be seen that fructose is slightly

GLYCOGEN FORMATION IN THE LIVER OF THE RAT

(The animals received no food for twenty-four to forty-eight hours prior to the experiments. This reduces the glycogen content of the liver to 0.1 to 0.2 per cent. The amount absorbed is given in grams per 100 Gm. rat. Liver glycogen is expressed as per cent.)

| Substance fed. | Hours after feeding. | Amount absorbed. | Liver glycogen. | References. |
|-------------------------|----------------------|------------------|-----------------|-------------|
| Glucose..... | 4 | 1.06 | 5.3 | (13) |
| Fructose..... | 4 | 0.54 | 5.7 | (13) |
| Galactose..... | 4 | 1.10 | 1.2 | (13) |
| Dihydroxyacetone..... | 4 | 0.69 | 4.0 | (15) |
| Glyceric aldehyde..... | 3 | | 0.8 | (16) |
| Sarcosolactic acid..... | 3 | 0.11 | 1.2 | (17) |
| Methylglyoxal..... | 3 | | 0.5 | (16) |
| Pyruvic acid..... | 3 | | 0.8 | (16) |
| Alanine..... | 3 | 0.21 | 0.6 | (18) |
| Glycerol..... | 4 | | 2.4 | (19) |

superior to glucose in its ability to form liver glycogen in the rat and that galactose is very much inferior. In interpreting these figures it must be remembered that in rats fructose is absorbed half as fast and galactose at about the same rate as glucose.

A number of other sugars were tested for their ability to form liver glycogen in rats. Pentoses formed no liver glycogen.¹⁴ The triose dihydroxyacetone ($\text{CH}_2\text{OH.CO.CH}_2\text{OH}$) formed liver glycogen quite rapidly. Another triose, glyceric aldehyde ($\text{CHO.CHOH.CH}_2\text{OH}$) gave only 0.8 per cent liver glycogen in three hours but the racemic form was used. Of the two isomers probably only one gives rise to glycogen deposition in the liver. Both dihydroxyacetone and glyceric aldehyde are assumed to be intermediaries in carbohydrate metabolism.

Several substances of noncarbohydrate nature can be converted to liver glycogen. The most important of these are listed in the preceding table. The dextrorotatory form of lactic acid (which belongs to the *l*-series, however) causes glycogen deposition in the liver when fed to rats, while the levorotatory isomer (or *d*-lactic acid) does not form liver glycogen. Methylglyoxal is converted to lactic acid by an enzyme present in most tissues and glycogen formation from this substance may therefore take place by way of lactic acid. Methylglyoxal is quite toxic and this may explain the lower yield of glycogen as compared to lactic acid.

Pyruvic acid and alanine form similar amounts of glycogen. Pyruvic acid is formed by oxidative deamination from alanine and is presumably reduced to lactic acid before glycogen synthesis takes place. Feeding glutamic acid, and to a lesser extent glycine, likewise causes glycogen deposition in the liver.¹⁸ Part of the protein molecule is thus available for glycogen formation in the liver. Glycerol, which constitutes about 10 per cent of the fat molecule, is another important source of liver glycogen; in feeding experiments on rats it formed 2.4 per cent in four hours.

The higher fatty acids have been tested repeatedly for their ability to form liver glycogen, but the results were uniformly negative. Acetic, butyric, valeric and caproic acids likewise fail to form liver glycogen, whereas propionic acid gives rise to glycogen deposition in the liver.²⁰

The livers of animals on a protein-fat diet contain considerable glycogen which is formed entirely from noncarbohydrate sources. During prolonged starvation, the liver glycogen is generally reduced to a low level and the blood sugar may range somewhat below normal; but the liver glycogen never becomes completely exhausted. Amino acids and glycerol derived from the catabolism of body protein and fat, respectively, and lactic acid derived from muscle glycogen, serve as the materials for glycogen synthesis in the liver of the fasting organism.

When the isolated liver of a mammal or frog is perfused with blood or Ringer's solution containing glucose, glycogen synthesis can be demonstrated. Little is known about the actual mechanism of glyco-

gen synthesis, except that the process does not occur anaerobically. The glycogen content of the liver of an animal in the postabsorptive state may show very marked individual variations; in rabbits and dogs it may vary anywhere between 3 and 12 per cent. The frog liver contains much more glycogen in the fall and winter than during the summer months.

Glycogenolysis in Liver.—Liver, muscle and other tissues contain a diastatic enzyme which can be separated from the cells. This isolated diastase forms reducing sugars from glycogen or starch, and the pH optimum in the absence of chloride ions is about 6.2. When chloride ions are present, diastatic activity is markedly increased, and the pH optimum is between 7 and 7.4.²¹ This corresponds to the conditions under which the diastase may be expected to act in the liver cell. A diastase is also found in the blood and in urine. The origin of the blood diastase is not entirely clear, but it has been shown that traumatization during operation or pathologic lesions of the pancreas or obstruction of the main pancreatic duct lead to a marked rise in blood and urinary diastase.

In recent experiments with blood and urinary diastase Somogyi²² pictures the hydrolysis of starch or glycogen as follows: Glycogen-dextrins-anhydrosugars-isomaltose-maltose-glucose. Possibly the same intermediates are formed when glycogen is acted upon by the cell diastases. A trisaccharide has been described as the product of hydrolysis of glycogen by muscle diastase, but its position in the scheme given above is not yet clear.²³

The point to be emphasized is that the end-product of hydrolysis of glycogen in the liver is glucose. The lactic acid formed by liver tissue kept outside the body seems to be produced chiefly by the glycolytic activity of the blood contained in the liver, because liver tissue washed free of blood forms only negligible amounts of lactic acid.²⁴ The liver seems to lack a glycolytic enzyme system such as is found in muscle and in a number of other tissues, and it is for this reason that the breakdown of glycogen progresses only to the glucose stage.

Several factors may be shown to influence the diastatic activity of the liver. When the liver cells are killed by freezing, or when their structure is destroyed by grinding, hydrolysis of glycogen is very markedly increased. Under these conditions, a frog liver kept at 20° C. may lose 1 per cent of glycogen per hour. It is obvious that if the same rate of glycogenolysis prevailed *in vivo*, the liver would lose all its glycogen in a short time. Since this does not happen in the intact liver, one must assume that only part of the enzyme is active, and that the greater part of it is present under such conditions that it is unable to act on its substrate glycogen. It has been suggested that part of the enzyme is adsorbed on protein and thus removed from the sphere of action. An increase in the rate of hepatic glycogenolysis would be brought about by a liberation of the adsorbed enzyme.²⁵

Diastatic activity of the intact liver can be increased in several

ways. If the blood supply of the mammalian liver is clamped off for a short time, a rapid breakdown of glycogen takes place and the blood sugar concentration rises. Asphyxia of the whole animal has the same effect, and the hyperglycemia observed after a severe hemorrhage is in part due to insufficient oxygenation of the liver. Surface active substances, such as ether, ethanol and other alcohols accelerate glycogenolysis in the isolated frog liver when added to the perfusion fluid.

The most important physiologic agent having an accelerating effect on hepatic glycogenolysis is epinephrine. When the isolated frog liver is perfused with Ringer's solution containing epinephrine in a concentration of 1 to 1,000,000, an increased amount of sugar appears in the perfusion fluid. This effect is reversible; that is to say, on changing back to Ringer's solution without epinephrine, sugar formation returns to the basal level.²⁶ In mammals, epinephrine, whether injected or released by the adrenals, likewise causes an acceleration of hepatic glycogenolysis.

The adrenal medulla is under the control of the sympathetic nervous system, and a release of epinephrine is brought about reflexly as the result of various stimuli. After inactivation of the adrenal medulla by cutting its nerve supply, many previously powerful glycogenolytic stimuli have only a feeble effect. Claude Bernard's *piqûre* is a case in point. When the floor of the fourth ventricle is punctured in the neighborhood of the tenth nucleus, certain sympathetic tracts are stimulated, and this causes such an intensive glycogenolysis in the liver that hyperglycemia and glycosuria persist for several hours. After inactivation of the adrenal medulla *piqûre* has only a slight effect on blood sugar and no longer produces glycosuria.²⁷

A release of epinephrine is brought about under a variety of conditions; such as emotional excitement, pain, stimulation of sensory nerve endings, especially those in the peritoneal cavity, exposure to cold, asphyxia, hemorrhage and administration of certain drugs. It is of special importance for blood sugar regulation that a release of epinephrine from the adrenal medulla is also brought about by hypoglycemia.²⁸ That this is actually a protective measure on the part of the organism, is shown by the fact that animals with inactivated adrenals are hypersensitive to insulin. One fourth to one sixth of the usual dose of insulin will produce severe hypoglycemic convulsions in rabbits with inactivated adrenals.

Apart from epinephrine, other hormones may enhance the glycogenolytic activity of the liver, but their action on carbohydrate metabolism has not yet been analyzed sufficiently. After prolonged thyroid administration, the glycogen content of the liver falls to a low level. This may mean that glycogenolysis proceeds at such a rapid rate that glycogen synthesis can no longer keep pace with it; but other explanations are also possible. Attention has recently been directed to the anterior lobe of the hypophysis as the site of production of a hormone concerned with carbohydrate metabolism. Extracts of the anterior

lobe, when administered for some days, produce glycosuria in normal animals. Further work is needed to elucidate the mechanism of this glycosuric action.

While there are a variety of agents which enhance glycogenolysis in the liver, we know at present of only one physiologic agent which has the opposite effect, namely, insulin, the internal secretion of the islet tissue of the pancreas. The evidence for an inhibitory action of insulin on hepatic glycogenolysis may be summarized as follows: (a) Following removal of the pancreas from a dog—the operation may be performed in two stages, so that anesthesia is not required for the final operation—the blood sugar begins to rise immediately and after a few hours the glycogen content of the liver is reduced to a low level. If now a large amount of glucose is administered, a temporary rise in liver glycogen may be demonstrated.²⁹ This seems to indicate that the disturbance due to lack of insulin is not in the synthetic but in the glycogenolytic process; *i. e.*, the diabetic liver, though able to store glycogen, is unable to retain it owing to an increased rate of glycogenolysis. (b) When insulin and glucose are administered to a depancreatized dog in suitable dosage, the glycogen content of the liver rises and may be maintained at a normal level.³⁰ Insulin aids indirectly in the storage of glycogen by inhibiting hepatic glycogenolysis, though the possibility cannot be excluded that it also enhances the synthetic process. (c) The perfused liver of a depancreatized frog forms three to six times as much sugar as that of a normal frog, while the liver of a frog which has been given an insulin injection on the preceding day produces less sugar than the liver of a normal frog.³¹ The decrease in liver glycogen which is regularly observed in rabbits and rats at a certain time interval after epinephrine injection can be prevented if insulin is injected simultaneously with the epinephrine.³²

One of the early observations in regard to the action of insulin on normal fasting animals was that liver glycogen diminishes if hypoglycemia persists for any length of time. This would be inconsistent with the view that insulin inhibits hepatic glycogenolysis, were it not that hypoglycemia in itself is a powerful stimulus for glycogenolysis, so that the inhibitory effect of insulin is overcome. Furthermore, hypoglycemia leads to an increased discharge of epinephrine from the adrenal medulla, another factor which causes glycogenolysis in the liver. Since, however, the liver glycogen also diminishes when insulin is injected into adrenalectomized animals, this factor seems to be of secondary importance only.³³

Ergotoxine, an alkaloid extracted from ergot, which paralyzes most sympathetic nerve endings, inhibits the glycogenolytic action of epinephrine both in frog and mammalian liver.

Glycogen Formation in Muscle.—The glycogen which is constantly being used up in muscle is replaced by synthesis from blood sugar. During prolonged muscular activity, when the demand for blood sugar is high, the liver glycogen diminishes and there occurs, under these

conditions, a transfer of liver glycogen to muscle glycogen by way of blood sugar.

During glucose absorption from the intestine, the glycogen content of the muscles increases. In experiments on rats, 25 per cent of the glucose absorbed in four hours was stored as glycogen in the total muscle mass of the body, as compared to 18 per cent in the liver.³⁴ Other monosaccharides also lead to the deposition of muscle glycogen, but since the liver can convert them to glucose, this does not prove that they are able to form muscle glycogen directly. Any substance convertible to glycogen or glucose in the liver is a potential source of muscle glycogen.

Glycogen deposition in muscle from blood sugar is greatly enhanced by insulin. In order to produce a definite rise in muscle glycogen in depancreatized dogs by means of glucose injection, it is necessary to supply insulin at the same time. Eviscerated animals do not store muscle glycogen unless insulin is injected.³⁵ Moreover, if glucose and insulin are administered to a normal animal, the muscles deposit more glycogen than if the same quantity of glucose is given without insulin. In the former case, 36 per cent of the glucose given was found as glycogen in the muscles of the body as compared to 25 per cent of the latter case (see the accompanying table). Obviously the animal's own

CARBOHYDRATE BALANCE IN RATS AFTER FOUR HOURS OF GLUCOSE ABSORPTION

| | Oxidized. | Deposited as liver glycogen. | Deposited as gly- cogen in rest of body. | Excreted in urine. | Blood sugar. | Respira- tory quotient. |
|--------|-----------|------------------------------------|--|-----------------------|-----------------|-------------------------------|
| A..... | 44 | 18 | 25 | 0 | 158 | 0.88 |
| B..... | 49 | 6 | 36 | 0 | 72 | 0.90 |
| C..... | 39 | 23 | 15 | 6 | 198 | 0.82 |

(A, No injection; B, insulin injected; C, epinephrine injected. Injections were made at the time of sugar feeding. The values are expressed as per cent of the amount absorbed.) (Data from Cori and Cori.^{15, 46})

pancreas did not supply enough insulin to allow glycogen formation in muscle to proceed at its maximum rate, and hence the process could be accelerated by supplying an additional amount of insulin.

Since the insulin secreted by the pancreas passes through the liver by way of the portal circulation before it reaches the other tissues of the body, this organ has probably all the insulin it can use. It is therefore not surprising that glucose and insulin injected into a normal animal do not cause more glycogen to be deposited in the liver than when glucose alone is given. As a matter of fact, if the experimental conditions are such that the same quantity of glucose is available both to the normal and the insulinized animal, the latter will deposit an

extra amount of muscle glycogen at the expense of liver glycogen (see the preceding table).

In the liver of the diabetic animal insulin promotes glycogen deposition, but there is no means of deciding whether this is due wholly to an inhibition of glycogenolysis, or to an acceleration of glycogen synthesis as well. In muscle, where an inhibiting effect of insulin on glycogenolysis has not been demonstrated, it is very probable that insulin actually enhances the deposition of glycogen.

Muscle possesses another mechanism for glycogen synthesis which is connected with the contraction process and seems to be independent of the supply of insulin. If muscle of a depancreatized animal is stimulated, causing a breakdown of glycogen, a resynthesis of glycogen takes place during a subsequent period of rest, presumably from the lactic acid and hexosemonophosphate accumulated in muscle during the contraction. However, stimulated muscle must draw on blood sugar in order to regain its initial glycogen content. It is during the late phase of recovery that a difference between normal and diabetic muscle becomes apparent. The diabetic muscle regains its glycogen at a slower rate, because the rate of conversion of blood sugar to glycogen is dependent on the supply of insulin.³⁶

Glycogenolysis in Muscle.—It is difficult to assign any "normal" value to the glycogen content of muscle, because not only is there a considerable variation from species to species, but different muscles of the same animal do not contain the same amount of glycogen. In rats, the gastrocnemius muscle contains about 0.6, and in rabbits 1 per cent glycogen. In dogs, values up to 1.8 per cent have been reported.* On the assumption that the average glycogen content of human muscle is 1 per cent, and that the muscles constitute about 45 per cent of the body weight, a man of 70 kilos would contain 315 Gm. of glycogen in his muscles. This is more than is contained in the liver. Yet this considerable glycogen store is not directly available as blood sugar, because the end-product of glycogenolysis in muscle is lactic acid.

The mechanism of glycogenolysis in muscle has been elucidated in a remarkable manner. It is possible to prepare a cell-free extract of muscle, which is capable of forming lactic acid from added glycogen. This muscle extract contains a diastatic enzyme and the glycolytic enzyme complex. The latter can be separated into a heat-labile component, the enzyme proper, and into a heat-stable, dialyzable component, the so-called "coenzyme of fermentation." The coenzyme consists of inorganic phosphate, magnesium and adenosinetriphosphate. In the absence of the coenzyme or any of its components, lactic acid formation does not take place.³⁷

* Many values given in the literature are erroneous because the animals were killed before the muscle was taken for analysis, a procedure which causes a considerable loss of glycogen. In order to obtain correct values it is necessary to remove the muscle from the living animal (anesthetized with amytal or otherwise suitably prepared).

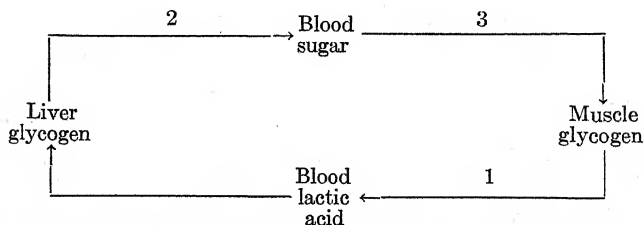
The first step in lactic acid formation in muscle consists in a splitting of glycogen by the diastatic enzyme. The split products of glycogen are esterified with phosphoric acid. Two esters are formed, namely, glucose-6-phosphoric acid, or *Embden* ester, and fructose-1:6-diphosphoric acid, or *Harden-Young* ester. The former ester is a constituent of normal muscle;³⁸ the latter ester, owing to its great lability, does not accumulate in intact muscle under physiologic conditions and is presumably the immediate precursor of lactic acid.

Muscular contraction involves a splitting of glycogen to lactic acid, but we are concerned here primarily with resting muscle and not with the muscle as a machine. Lactic acid formation is not necessarily restricted to the contraction process. Resting muscle is presumably forming some lactic acid all the time, but lactic acid does not accumulate in the presence of oxygen, and hence its formation cannot be detected. When amphibian muscle is kept anaerobically, lactic acid accumulates at a steady rate for many hours; and it has been suggested that the same rate of lactic acid formation prevails in oxygen.

Epinephrine, when added to frog muscle suspended in Ringer's solution, causes a decrease in glycogen³⁹ and at the same time a rise in hexosemonophosphate and lactic acid.⁴⁰ A concentration of 1:100 millions was found still to be effective in thin muscles. In contrast to the liver, the glycogenolytic effect of epinephrine on muscle cannot be inhibited by ergotoxine or insulin.

In mammals, epinephrine, whether injected or discharged by the adrenals, causes a breakdown of muscle glycogen and an accumulation of hexosemonophosphate and lactic acid.⁴¹ Part of the lactic acid formed in muscle diffuses into the blood stream and is transformed to glycogen in the liver. Muscle glycogen is thus a potential source of blood sugar, and one of the functions of epinephrine in the body is to make muscle glycogen available as blood sugar through the mechanism just described.

Glucose is capable of going through a complete cycle in the body; it may be in turn, blood sugar, muscle glycogen, lactic acid, liver glycogen and again blood sugar. This is depicted in the following diagram.



Epinephrine, by accelerating reactions 1 and 2 of this cycle, causes lactacidemia and hyperglycemia; while insulin by accelerating reaction 3 causes hypoglycemia. Some evidence is also available that insulin retards reaction 2 and epinephrine reaction 3.

Carbohydrate Oxidation.—Glycogen formation and carbohydrate oxidation are the two most important mechanisms for disposal of sugar in the body. They account for 90 per cent of the ingested glucose utilized by an animal which has previously been fasted. About one half of the absorbed sugar is oxidized, while the other half is found as glycogen in liver, muscle and other tissues of the body. If the animal is not in the fasting state at the time of glucose administration, part of the sugar may be transformed to fat, as set forth in the next section.

The *respiratory quotient*, $\frac{\text{vol. CO}_2}{\text{vol. O}_2}$, is generally used as an index of the nature of the foodstuffs undergoing oxidation. It may be seen that the quotient for oxidation of carbohydrate is $\text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 = 6\text{CO}_2 + 6\text{H}_2\text{O} = 6\text{CO}_2/6\text{O}_2 = 1$; while the quotient for fat, as may easily be calculated, is 0.7. Protein oxidation in the body yields a quotient of 0.8. The amount of protein oxidized can be calculated from the urinary nitrogen excretion, and by deducting from the total respiratory quotient the CO_2 and O_2 corresponding to protein oxidation, one obtains the nonprotein respiratory quotient, which indicates the proportion of fat and carbohydrate oxidation. In a normal organism, the great majority of the nonprotein respiratory quotients lie between the limits of those of fat and carbohydrate.

After a period of starvation, the quotient is generally reduced to a low level; *i. e.*, the organism lives mostly at the expense of its own fat and protein, while carbohydrate oxidation is reduced to a minimum. If now carbohydrates are administered the quotient rises, because carbohydrate oxidation replaces that of fat and protein.

In man, fructose, galactose* and dihydroxyacetone produce a greater and more rapid rise of the respiratory quotient than glucose.⁴² Several explanations have been offered for this observation. One is that these sugars are actually oxidized more rapidly and to a larger extent than glucose. Another is that the quotient, under these conditions, is not a true indication of the foodstuffs oxidized, because some of these sugars lead to lactic acid formation in blood and tissues.⁴³ The lactic acid causes a liberation of CO_2 from blood bicarbonate, which, added to the CO_2 arising from metabolism, produces a rise of the quotient. A third is—and this is claimed for fructose only—that this sugar is converted to a larger extent to fat than is glucose (conversion of sugar to fat increases the quotient). There seem to be no experimental data available at present which would lead to the acceptance of one of these theories in preference to the others. Furthermore, it has not yet been shown whether the sugars mentioned are converted to glucose in the liver before they are oxidized by the tissues or whether they are oxidized directly.

In man or dog the O_2 intake rises when sugar is fed, an effect

* In man only small amounts of galactose are excreted in the urine after ingestion of this sugar, whereas in dogs, rabbits and rats up to 50 per cent are excreted. This indicates that galactose metabolism is different in man than in the laboratory animals.

called the *specific dynamic action* of carbohydrate. It may amount to 5 to 20 per cent of the basal O_2 intake. The specific dynamic action depends on the condition of the subject: It is small or absent when sugar is fed after prolonged starvation. The plethora of an easily oxidizable substance in the tissues is usually regarded as the cause of the rise of calorie production.

The quotient of depancreatized dogs is generally between 0.68 and 0.72,* in spite of an excess of sugar present in the blood, and if glucose is administered, the quotient does not show the typical rise which is observed in the normal animal. These results have also been obtained on isolated tissues. Muscle and kidney tissue of depancreatized dogs show a quotient of 0.7 to 0.75; and addition of glucose fails to produce a rise in the quotient, whereas the same tissues removed from a normal dog yield quotients between 0.75 and 1 and respond with a definite rise in the quotient when glucose is added.⁴⁴

The depancreatized dog, in order to metabolize an adequate quantity of glucose, needs insulin; and if this is supplied, one finds a pronounced rise in the respiratory quotient. In normal man and animals, injection of insulin also causes a rise in the quotient. If glucose plus insulin is administered, one obtains a greater rise in the quotient than when the same amount of glucose is given without insulin. The situation here is similar to that of glycogen formation in muscle; *i. e.*, the insulin supplied by the animals' own pancreas is insufficient to allow carbohydrate oxidation to proceed at its maximum.

The evidence just presented indicates that insulin accelerates carbohydrate oxidation in the tissues. Epinephrine, on the other hand, may be shown to diminish carbohydrate oxidation under certain experimental conditions. It is true that the respiratory quotient rises after epinephrine injection, but this is due to the fact that the excess of lactic acid formed in muscle liberates CO_2 which is blown off in the lungs. Indeed, one finds a marked decrease in the bicarbonate content of the blood after epinephrine injection; and under such conditions the respiratory quotient is not a true index of the foodstuffs oxidized in the body. This applies particularly to metabolism periods of short duration. If the metabolism after epinephrine injection is measured for several hours, *i. e.*, until the original acid-base balance is reestablished, the error introduced in the respiratory quotient by the blowing off of CO_2 can be avoided. During a period of four hours rats receiving glucose plus epinephrine oxidized less sugar than rats receiving glucose alone, the average quotient for the whole period being 0.82 in the former and 0.88 in the latter case. In rats in the postabsorptive state epinephrine injections caused an increase in carbohydrate oxidation. A carbohydrate balance showed, however, that this occurred at the expense of muscle glycogen and not of blood sugar.⁴⁵

* Dogs which survive pancreatectomy for several weeks show a rise of the respiratory quotient from 0.7 to 0.85 shortly before death. A satisfactory explanation for this apparent resumption of carbohydrate oxidation has not yet been found. (Hédon, L., *Ann. Physiol.*, 10, 905, 1934).

A pronounced inhibitory effect of epinephrine on carbohydrate oxidation is observed in anesthetized animals. When cats under amytal anesthesia are given a constant intravenous injection of glucose and epinephrine is added to the injection fluid, utilization of glucose may be suppressed completely, so that the amount of glucose excreted in the urine equals that injected. The respiratory quotient under these conditions is reduced to 0.72 and remains at this low level in spite of a continuous supply of glucose. Both amytal and epinephrine, when acting alone, diminish carbohydrate oxidation somewhat, but the combined effect of the two is much greater than would be predicted on the basis of simple summation of their separate effects, which may be due to a potentiation of the epinephrine effect by amytal.⁴⁶

What is the mechanism of carbohydrate oxidation in the tissues? Are the first steps of degradation of the sugar molecule the same under oxidative and anoxidative conditions? Or does oxidation follow an entirely different path? Some evidence has been adduced recently that muscle is able to oxidize lactic acid. Frog muscle poisoned with iodoacetate loses its ability to form lactic acid and, at the same time, the respiratory quotient drops from a value near unity to 0.75. If now lactate is added to such a muscle, the quotient rises.⁴⁷ On these grounds one could assume that glucose has to be broken down to lactic acid in the tissues before it can undergo oxidation, and insulin might act by accelerating the oxidation of lactic acid. This assumption is rendered improbable by the fact that insulin injections do not increase the rate of removal of lactic acid from the blood.⁴⁸

Another possibility is that insulin accelerates the transformation of glucose to lactic acid. The following experiments are relevant in this respect. A cell-free and carbohydrate-free extract of mammalian muscle was prepared which formed lactic acid from added glycogen, but formed practically no lactic acid from added glucose.⁴⁹ Addition of hexokinase, an activator extracted from yeast, enabled the extract to form lactic acid at a greater rate from glucose than from glycogen. Insulin was, however, unable to replace hexokinase in this system, since its addition to the extract did not lead to lactic acid formation from glucose. There is thus no evidence available which would show that insulin aids in the oxidation of lactic acid or in the transformation of glucose to lactic acid.

Before glucose is split to lactic acid, it is esterified with phosphoric acid. It is possible that oxidation sets in at this point, which would mean a common initial path in oxidative and anoxidative degradation. The hexosephosphate esters are more easily oxidized in the test tube and by certain biological systems than is glucose.⁵⁰ In hexosediphosphate, in particular, the sugar (fructose) is present in an unstable ring form; *i. e.*, as the $< 2,5 >$ lactal form.⁵¹ The unsaturated free radicle would exist for a short time when the ring opens and this highly reactive form might undergo oxidation with greater ease than the relatively stable ring form of α , β -glucose. Combination with phosphoric acid might thus be a mechanism by means of which the sugar molecule is

activated not only for dissociation into smaller molecules, *i. e.*, lactic acid, but also for oxidation. It is difficult to test this hypothesis experimentally, because hexosephosphates, when added to surviving tissues, do not penetrate into the cells. An accumulation of hexosediphosphate in muscle has so far not been observed under physiologic conditions. Apparently the ester, owing to its great lability, is decomposed as rapidly as it is formed. In stimulated muscle, previously poisoned with iodoacetate, hexosediphosphate and monophosphate esters accumulate, but the respiratory quotient of such a muscle is reduced to 0.75.⁴⁷ Unless one makes the assumption that iodoacetate besides inhibiting lactic acid formation, also prevents oxidation of hexosephosphate esters, this experiment would disprove the hypothesis that the diphosphate or monophosphate ester is an intermediary in glucose oxidation.

It is conceivable that glucose might be activated in other ways. Numerous workers have attempted to demonstrate some form of active glucose in blood and tissues, but so far these efforts have failed. An analogy is often drawn between the biological behavior of sugar and the behavior of sugar in an alkaline solution. When exposed to high alkalinity, sugars become unstable: They develop strong reducing intensity and become autoxidizable. Polymerization and depolymerization take place and the sugars are, in part, converted to lactic acid with intermediate formation of methylglyoxal. Shaffer⁵² concludes from his studies that this instability is primarily the consequence of salt formation of the weakly acid sugars, and that sugar ions are the first active forms of sugar which appear in alkaline solution. By aeration of sugar in alkaline solution, molecular oxygen is activated through the formation of a sugar peroxide. Such a system is then able to oxidize other substances which are not directly attacked by molecular oxygen. It is attractive to picture the behavior of biological systems in a similar manner.

It seems that, as far as experimental evidence goes, we are left entirely in the dark as to the actual mechanism of glucose oxidation in the tissues. The only well-established fact seems to be that insulin accelerates this oxidation. Since sugar produced by enzymatic hydrolysis of glycogen may be present in an active form, it has been suggested that insulin promotes primarily glycogen deposition in muscle, and that the resulting increase in oxidation is due to the cleavage of this glycogen. With the enzyme systems existing in muscle it seems more probable, however, that cleavage products of glycogen are led into the lactic acid path; so that muscle glycogen would be oxidized by way of lactic acid rather than glucose.

Fat Formation.—In certain species of animals, especially in swine and geese, feeding a diet consisting primarily of carbohydrate and enough protein to meet the minimum requirements, leads to deposition of body fat. The fat formed from carbohydrate is of hard consistency and consists mainly of the triglycerides of palmitic and stearic acid,

in contrast to the "soft pork" which is found when hogs are fed vegetable fats containing a large proportion of oleic acid.⁵³ The production of a mixture of equal parts of tristearin and tripalmitin from glucose may be written as follows: $13\text{C}_6\text{H}_{12}\text{O}_6 = \text{C}_{55}\text{H}_{104}\text{O}_6 + 23\text{CO}_2 + 26\text{H}_2\text{O}$. From this it may be calculated that 274 Gm. of glucose will yield 100 Gm. of fat, 101 Gm. of CO_2 and 47 Gm. of H_2O . This is very similar to the calculation of Bleibtreu,⁵⁴ who on the basis of C, H, and O analyses of pork, calculated that 270 Gm. of glucose yield 100 Gm. of fat, 115 Gm. of CO_2 and 55 Gm. H_2O .

In terms of heat, 1025 calories of glucose would yield 940 calories of fat; *i. e.*, one is dealing with an exothermic reaction. It may be seen that CO_2 is formed in the process of transformation of carbohydrate to fat. Obviously, if we knew the amount of CO_2 formed, we could calculate the amount of fat formed from the above equation. However, as long as the respiratory quotient is unity or below, there is no means of distinguishing between the CO_2 arising from combustion and the CO_2 formed in the sugar-to-fat transformation; and hence one cannot say at what level of the respiratory quotient this transformation sets in. It is possible, for instance, that a nonprotein quotient of 1 represents the algebraic sum of, say, a true combustion quotient of 0.95 and some CO_2 evolved in the sugar-to-fat transformation.

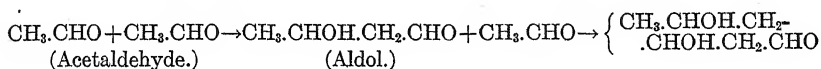
When the nonprotein quotient rises above unity, the CO_2 formed above a nonprotein quotient of 1 may be used for a calculation of the amount of fat formed from carbohydrate. In a young hog of about 13 kilos, the respiratory quotient rose to 1.4 three hours after the ingestion of 700 Gm. of starch and remained at this level for about twenty hours.⁵⁵ The production of fat, as calculated from the extra CO_2 formed, averaged 5.2 Gm. per hour or 125 Gm. per day, which corresponds to 1 per cent of the body weight. Of the calories contained in the starch, 56 per cent were converted to fat. Similar results were obtained on geese fed forcibly with carbohydrate. In other species the results are not so striking.

It is an everyday experience that certain individuals are more prone to deposit fat than others, and it is commonly supposed that the endocrine glands play an important rôle in this respect. As yet the influence of hormones on the sugar-to-fat transformation has not been studied extensively enough to form a clear picture.

After a large carbohydrate meal there occurs a filling-up of the glycogen stores in liver, muscle and other tissues. The capacity of the tissues to store glycogen is not unlimited; and at the same time there is a considerable individual variability in the maximum glycogen level attained in liver and muscle. With a continued influx of glucose into the blood, carbohydrate oxidation rises more and more and the O_2 consumption increases above the basal level. Soon oxidations are carried on almost exclusively at the expense of carbohydrate, and the nonprotein quotient is close to unity, while the rate

of glycogen deposition begins to fall off. It is at this point that fat formation must set in in order to dispose of the continued supply of carbohydrate. Once this mechanism has been set in motion, it may continue for some time even during the postabsorptive period, because part of the newly formed glycogen is broken down again. It is not surprising that with so many variables, great differences are observed in the ability to form fat from carbohydrate, not only between different species, but also between individuals of different age and sex of the same species.

In regard to the mechanism of fat synthesis from carbohydrate, the most plausible theory proposed is that of aldol condensation, which may be presented as follows:



In this way, even-numbered carbon chains may be built up, which, by a process of reduction and by oxidation of the terminal aldehyde group, may be converted to fatty acids.

Acetaldehyde occurs only in traces in the animal organism. Theoretically, it might be formed from pyruvic acid by a process of decarboxylation; thus, $\text{CH}_3.\text{CO}.\text{COOH} = \text{CH}_3.\text{CHO} + \text{CO}_2$. This is what happens during alcoholic fermentation of yeast; but the animal tissues seem to lack the enzyme which carries out this decarboxylation. It is conceivable that other aldehydes are formed in the tissues from which fat is synthesized.

The synthesis of fat from carbohydrate is a remarkable feat of the organism and a further elucidation of the mechanism is of considerable interest. Naturally the question arises whether the reverse reaction can be carried out; namely, the conversion of fat to carbohydrate. This is a subject on which there is considerable discussion at the present time. In germinating seedlings such a conversion has been demonstrated by various methods. It may be shown by chemical analyses that the fat content of the castor bean diminishes during germination and that, at the same time, the carbohydrate content increases. The respiratory quotient of the bean during germination has been shown to be very low (below 0.5), as is to be expected if a substance poor in oxygen (fat) is converted into one rich in oxygen (carbohydrate).⁵⁶

The conversion of the glycerol moiety of the fat molecule to glucose in the animal organism has been discussed previously. When fatty acids are fed to normal animals, glycogen deposition in liver and muscle cannot be demonstrated. Similarly, the administration of fatty acids or lecithin to phlorhizinized dogs does not increase the sugar excretion in the urine;⁵⁷ while administration of certain amino acids and a variety of other substances causes an excretion of extra sugar (see p. 570). It is difficult to see why fatty acids, if they were

convertible to glucose in the diabetic organism, should behave differently from amino acids. A crucial experiment which would prove a conversion of fatty acids to carbohydrate in the animal organism has not been performed.

Kidney Excretion.—During the passage of blood through the kidney, part of the glucose is filtered through the glomerular membrane and is reabsorbed in the tubules. The reabsorption mechanism is not absolutely perfect, so that small amounts of glucose escape into the urine. On an average, 140 mg. of glucose are found in a twenty-four-hour urine sample of normal men.⁵⁸ Even during a prolonged period of starvation some glucose is found in the urine, though the amount is less than in an individual on a normal diet. Since about 200 liters of blood plasma are cleared in the glomeruli in twenty-four hours, containing about 200 Gm. of glucose, the amount of glucose which escapes reabsorption in the tubuli is only an insignificant fraction of the total amount reabsorbed. Ordinary qualitative tests fail to detect the small amounts of sugar present in normal urine.

If the glucose concentration of the blood passing through the kidneys exceeds a certain value (about 140 mg. per cent in normal individuals), the kidney threshold is reached; *i. e.*, the reabsorbing mechanism can no longer keep pace with filtration of sugar through the glomeruli and measurable quantities of sugar appear in the urine. The higher the blood sugar concentration rises, the more sugar is excreted in the urine.

The reabsorbing capacity of the tubular epithelium is not the same for different sugars. Fructose and pentoses show a much lower kidney threshold than glucose, and hence they are excreted in the urine even if their concentration in the blood is very low. In the frog kidney the following order in the rate of reabsorption is observed: Glucose > galactose > mannose > fructose > xylose > arabinose.⁵⁹ This reminds one of the different rates at which various sugars are absorbed by the intestinal epithelium. Though the order in the rate of absorption of the different sugars is not the same in the two organs, it emphasizes the fact that certain membranes possessing a specialized epithelial lining are selectively permeable for substances of very similar chemical configuration.

The glucoside phlorhizin inhibits the reabsorption of sugars in the tubules. The action is specific for sugars, since the reabsorption of amino acids is not affected. Under the action of phlorhizin, the rate of excretion of different sugars is very nearly the same.

Regulation of Carbohydrate Metabolism.—In the preceding sections were discussed the various metabolic processes which are involved in the production and in the disposal of blood sugar. We may now examine how these metabolic processes regulate the blood sugar concentration under different physiologic conditions. The first case to be considered is that of the fasting organism. At the beginning of the fasting period, the energy requirements are met in part by combustion of

carbohydrate, as shown by respiratory quotients of 0.8 to 0.9. As the fast proceeds, the respiratory quotient falls gradually until it reaches the fat-protein level. This means that the organism is sparing carbohydrate and is drawing largely on fat for its energy requirements. In a rat fasted for forty-eight hours, 90 per cent of the calories are furnished by fat and 10 per cent by protein, giving a respiratory quotient of about 0.72. Insulin production, which stimulates carbohydrate utilization, is presumably reduced to a minimum during fasting. The new formation of carbohydrate from protein and from the glycerol part of the fat molecule, balances the small amounts of blood sugar which continue to be used in the tissues. If the blood sugar concentration should fall to a dangerously low level, epinephrine is discharged; and this causes muscle glycogen to be broken down to a lactic acid from which the liver can form blood sugar.

When a fasting animal is allowed to exercise, so that the O_2 consumption rises to three to four times its normal value, the respiratory quotient remains at the fat-protein level.⁶⁰ The assumption is that the oxidation of fat furnishes the necessary energy for the reconversion of the lactic acid formed during contraction. If muscular activity were carried out at the expense of carbohydrate combustion, then one fifth of the lactic acid formed should be oxidized with reconversion of the remaining four fifths to glycogen. If this were the case during starvation, the glycogen stores of muscle would soon become exhausted.

Starvation is thus characterized by a rigid economy of the available glycogen stores in liver and muscle in the interest of blood sugar regulation and of muscular activity. A different kind of adjustment is necessary when there is a sudden increase in the supply of blood sugar, as for instance after an intravenous injection of glucose. In the first two to three minutes after such an injection, one observes a rapid fall in blood sugar concentration. This is not due to utilization of sugar, but is explained by the fact that the injected sugar diffuses into the tissues and distributes itself in a volume corresponding to about 50 per cent of the body weight. After this initial period of distribution, the rate of fall of blood sugar depends on the efficiency of glycogen formation and of oxidation, these being the chief metabolic processes which cause a removal of blood sugar. As stated previously, these metabolic processes are accelerated by insulin, and hence the shape of the blood sugar curve after administration of glucose becomes a measure of the functional activity of the islet tissue of the pancreas. If insulin secretion is slow, the blood sugar will remain abnormally high for a long period of time and sugar will escape into the urine. With rapid secretion of insulin the blood sugar will return rapidly to the normal level.

In clinical work, glucose is generally administered by mouth and the blood sugar determined at frequent intervals. In this case one must be aware of the fact that the rate of absorption of sugar and hence its rate of entrance into the blood are unknown and that it is

impossible to say to what extent variations in this factor may influence the shape of the blood sugar curve. This applies especially to pathologic conditions, where absorption is often disturbed. In normal men, after ingestion of 50 Gm. of glucose, the peak of the blood sugar curve is generally reached in thirty to forty-five minutes, and the values observed may vary between 120 and 200 mg. per cent in different individuals. In one and one-half to two hours the blood sugar has generally returned to the original level and, in certain individuals, it then falls below normal. This postprandial hypoglycemia is explained by an overshooting of insulin secretion. If at the time the blood sugar has returned to normal a second dose of 50 Gm. of glucose is given, the blood sugar does not rise as high as the first time, because the mechanism for the disposal of sugar has already been set in motion by the first sugar administration.⁶¹

The stimulus for insulin secretion seems to be the increased blood sugar concentration. Evidence for this contention is found in experiments in which glucose is injected directly into the pancreaticoduodenal artery, thus exposing the pancreatic tissue to a glucose concentration above normal. Such an injection is followed by a more rapid decline in blood sugar than occurs when the same quantity of glucose is injected into the femoral artery or portal vein.⁶² A nervous control of insulin secretion by way of the vagi has also been considered,⁶³ but since a denervated or transplanted pancreas regulates blood sugar just as well as one with intact innervation,⁶² the central vagal control cannot be very important.

In order to test the maximum capacity of an animal to dispose of sugar, timed intravenous injections have been made. In normal rabbits, dogs and man a rate of injection of 0.85 Gm. of glucose per kilo per hour can be tolerated for ten hours or longer without any excretion of sugar in the urine. If the rate of injection is raised slightly above the tolerance, so that some sugar is excreted in the urine, one finds that the glycosuria disappears after some time, the animal having attained a higher level of tolerance due to the continued stimulation of the high blood sugar concentration.⁶⁴ If the rate of injection is raised still further, glycosuria persists, but the excretion of sugar is not proportional to each increment in the rate of injection, owing to the fact that, within certain limits, the utilization of glucose increases with an increase in blood sugar concentration. The rate of glycogen deposition in liver and muscle is accelerated by a rise in blood sugar concentration.

Large doses of insulin raise the tolerance of an animal for intravenously injected glucose by about 20 per cent, while epinephrine injections have the opposite effect.

Insulin.—Attempts have been made to demonstrate a significant action of insulin on tissues *in vitro*. In experiments with the Warburg apparatus Takane⁶⁵ found an increase in the O_2 consumption and the respiratory quotient of the diaphragm muscle of rats when insulin

was added. A rise in the quotient is observed in intact animals when insulin is injected, and the rise is greater after glucose plus insulin than after glucose alone. This indicates that one of the functions of insulin in the body is to accelerate the oxidation of blood sugar in the tissues. Muscle glycogen is not broken down under the influence of insulin if hypoglycemia and convulsions are avoided.⁶⁶ Another well-recognized effect of insulin is an increased rate of glycogen deposition in muscle from blood sugar. These two effects account for the hypoglycemia after insulin injections, and for the larger amount of injected glucose which an animal is able to use under the influence of insulin. A third effect of insulin is an inhibition of hepatic glycogenolysis; this enables the liver to retain its glycogen, while in depancreatized dogs, in the absence of insulin, the liver glycogen disappears very rapidly.

In experiments on the isolated frog liver, it was noted that insulin added to the perfusion fluid had no effect but if the frog was injected with insulin on the day prior to the experiment, hepatic glycogenolysis was inhibited. This indicates that in cold-blooded animals insulin must remain for some time in the tissues before its effects become noticeable. The action of insulin in frogs has a definite temperature coefficient. Frogs kept at 6° to 8° C. develop hypoglycemic convulsions in one hundred and twenty to one hundred and forty hours; at 15° C., in sixty to seventy hours; at 20° C., in forty-three to forty-nine hours; at 25° C., in twenty-four to twenty-seven hours; and at 30° C., in fourteen hours.⁶⁷ If a frog could be maintained for any length of time at the body temperature of a mammal, convulsions would probably develop in the same length of time as in a mammal.

When the blood sugar is reduced to a low level (usually less than 40 mg. per cent) by injection of an overdose of insulin, the symptoms produced are very similar in all species of animals. In man, one observes hyperirritability, sweating, pallor, mental confusion, muscular weakness, tremors, a rise in blood pressure and basal metabolism and, finally, convulsive seizures. Some of these symptoms may be ascribed to the reflex discharge of epinephrine. After repeated convulsions, coma and death follow. In dogs kept for some days on a low water intake, the symptoms of hyperirritability are less pronounced and the animal may pass directly into insulin coma without showing convulsions.⁷⁰

It is of importance that hypoglycemic shock and convulsions also occur when the blood sugar is lowered by surgical removal of the liver in animals. The symptoms are therefore directly related to the blood sugar level and almost immediate relief is obtained in insulinized and in hepatectomized animals when glucose is injected intravenously. The rate of drop of blood sugar is markedly accelerated when insulin is injected into a hepatectomized dog,⁶⁹ indicating that insulin acts in the absence of the liver.

A large number of substances have been tested for their effective-

ness in alleviating hypoglycemic symptoms produced by insulin injections. Apart from glucose, mannose, dihydroxyacetone and, to a lesser extent fructose, glycerol and glucal were found to be active.⁷¹ In hepatectomized, in contrast to insulinized animals, dihydroxyacetone, fructose and glycerol (glucal has not yet been tested) do not relieve hypoglycemia, which indicates that the effectiveness of these substances depends on their conversion to glucose in the liver. All the other substances, which are known to be converted to glucose in the phlorhizinized animal fail to relieve insulin hypoglycemia, apparently because their rate of conversion to glucose is too slow to counteract the rapid fall in blood sugar.

Attempts to demonstrate an activation of the glucose molecule *in vitro* under the influence of insulin have failed so far. Perhaps insulin requires an activator which is supplied by the tissues. Crystalline insulin is a protein; under the assumption that the insulin-protein is merely a carrier for an active group, insulin was subjected to various chemical treatments with a view to splitting off an active group, but this has not been successful.⁶⁸ Trypsin digests the insulin-protein and the activity is lost with the destruction of the molecule. Apparently, the molecule as a whole is active, though there must undoubtedly exist a special chemical configuration to explain the rather unique action of insulin.

Epinephrine (familiarily known as adrenaline).—A subcutaneous injection of epinephrine in rabbits (0.2 mg. per kilo) causes a hyperglycemia of several hours' duration. An intravenous injection of epinephrine which has a marked effect on the circulatory system, causes only a short-lived hyperglycemia. The explanation for these observations lies in the fact that absorption of epinephrine from the subcutaneous tissue lasts several hours and that epinephrine, once it has entered the blood stream, is rapidly destroyed; hence a sudden intravenous injection has little after-effect. If epinephrine is injected slowly into a vein, the effect on carbohydrate metabolism persists as long as the epinephrine solution is flowing in; and if the rate of injection is within physiologic limits, conditions are directly comparable to epinephrine secretion by the adrenaline medulla (Fig. 39).

The maximal rate at which epinephrine is given off by the adrenal medulla during splanchnic stimulation has been determined in cats and was found to be 0.003 mg. per kilo per minute.⁷² This is associated with a considerable rise in blood pressure. However, the changes in carbohydrate metabolism after epinephrine injection are not dependent on a rise in blood pressure, since they may be produced at rates of intravenous injection which are considerably below the rate required to raise the blood pressure. Furthermore, a subcutaneous injection of epinephrine in laboratory animals is generally not followed by a rise in blood pressure but has, nevertheless, a marked effect on carbohydrate metabolism.

In rabbits, a continuous intravenous injection of 0.0001 mg. per

kilo per minute causes a definite rise in blood sugar and lactic acid; but in order to raise blood pressure, the rate of injection has to be increased five times. Liver and muscle appear to be equally sensitive to the glycogenolytic action of epinephrine. In man, blood sugar, lactic acid and basal metabolism rise when epinephrine is injected at a rate of 0.00005 mg. per kilo per minute (Fig. 40). Insulin has very little, if any effect on the increase in blood lactic acid produced by epinephrine.

The metabolic effects of epinephrine have been studied extensively in the rat. When 0.02 mg. per 100 Gm. were injected subcutaneously, there occurred first a decrease in liver glycogen. One hour after the

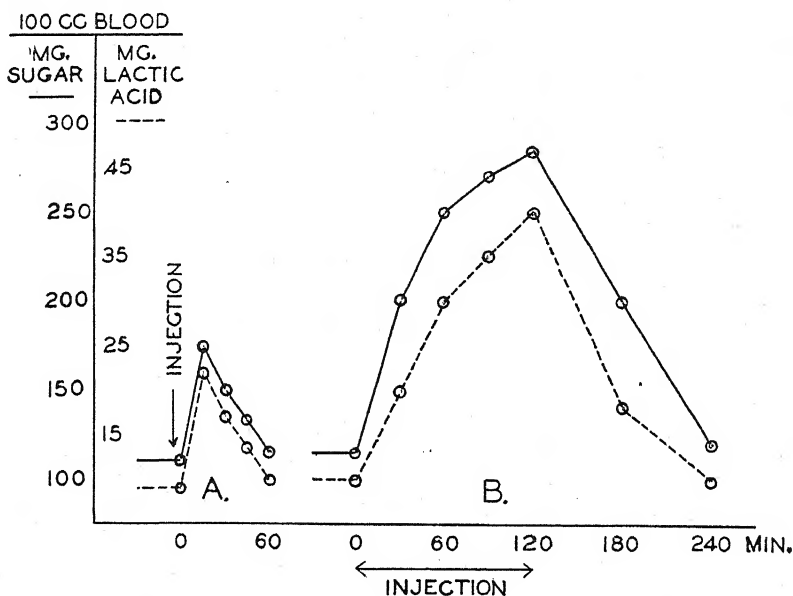


Fig. 39.—Effect of the same dose of epinephrine (0.03 mg. per kilo) on blood sugar and blood lactic acid of rabbits. *A*, Injected at once into vein. *B*, Injected slowly during two hours, rate 0.00025 mg. per kilo per minute. (Data from Cori, Cori, and Buchwald.⁷⁸)

injection, the liver glycogen returned to the original level and from then on it continued to rise reaching a maximum in about three hours. The blood sugar and blood lactic acid reached their maximum one hour after the injection, and returned to the basal level at the end of three hours (Fig. 41). The muscle glycogen was diminished at all periods. In experiments in which glycogen in the liver and in the rest of the body were determined separately it was found that the glycogen which was lost from the muscles was deposited in the liver; *i. e.*, a redistribution of glycogen had taken place. Muscle glycogen was broken down to lactic acid, the latter diffused into the blood stream and was carried to the liver where it was deposited as

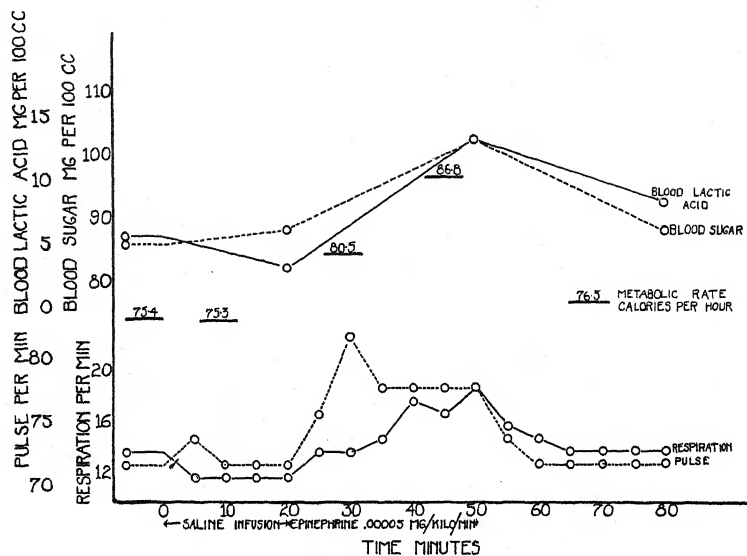


Fig. 40.—Metabolic changes produced in a normal man by injecting epinephrine intravenously at a rate of 0.00005 mg. per kilo per minute for thirty minutes. Note the rapidity with which the rise in metabolic rate sets in and the prompt return of the metabolic rate to the basal level after the cessation of the epinephrine injection. (Data from Cori, Cori and Buchwald.⁷³)

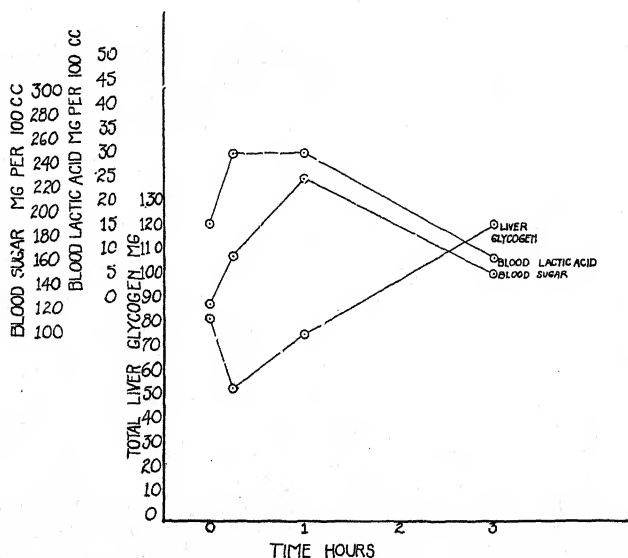


Fig. 41.—Effect of a subcutaneous injection of 0.02 mg. of epinephrine per 100 Gm. rat on liver glycogen, blood sugar, and blood lactic acid. Note the initial fall in liver glycogen followed by a rise. (Data from Cori and Cori.⁷⁴)

glycogen. At the same time liver glycogen was breaking down at a rate higher than normal, but this breakdown exceeded new formation only for a short time (fifteen minutes); later, the supply of blood lactic acid led to an excess of glycogen formation over glycogenolysis, although the latter process was still operative as shown by the elevated blood sugar level (see Fig. 41). In rabbits the same changes have been demonstrated,⁷⁵ but the time relations are different; the lowest liver glycogen was observed after one and one-half hours, the original level was reached after two and one-half hours and the highest level after five hours. In rabbits the effect of epinephrine on blood sugar and blood lactic acid is much more pronounced than in rats, dogs, cats or man.

Claude Bernard discovered that puncture of the floor of the fourth ventricle leads to a prolonged hyperglycemia and glycosuria. If the adrenals are removed, or if the splanchnic nerves are cut prior to the sugar puncture, the hyperglycemia is slight and lasts only for a short time. Apart from hyperglycemia, the sugar puncture also produces a rise in blood lactic acid, a decrease in liver and muscle glycogen and a rise in basal metabolism—effects which are characteristic of epinephrine action. Many drugs (morphine, strychnine, acetylcholine, nicotine, to name only a few) produce hyperglycemia by way of the adrenals. Ether and chloroform anesthesia produce less hyperglycemia in the absence of the adrenals than when the adrenals are intact; the residual hyperglycemia after adrenalectomy may be ascribed to the direct effect of the anesthetics on the liver.

Epinephrine produces in mammals a rise in O_2 consumption of 15 to 30 per cent.⁷⁶ This rise is not caused by muscular activity, since it occurs in rabbits after spinal transection and in frogs after destruction of the spinal cord. The following explanation has been given for the calorogenic action of epinephrine. Muscle glycogen is broken down to lactic acid and this lactic acid is reconverted to glycogen, largely in the liver but also in other tissues of the body. The reversion process requires energy because the heat of combustion of lactic acid is less than that of glycogen and hence an extra amount of foodstuff is burned.

In animals which are fasted for a long period of time, so that the glycogen reserves of the liver are very low, epinephrine injections still cause hyperglycemia, but to a lesser degree than in well-fed animals. The rise in blood lactic acid and in O_2 consumption is not influenced by preceding starvation; in a starved animal, the extra calories are furnished by fat combustion. After removal of the liver or of the abdominal viscera, epinephrine has no effect on the blood sugar, but it still causes a decrease in muscle glycogen and consequently raises blood lactic acid.⁷⁷ An increase in lactic acid has also been demonstrated in hepatectomized frogs and the calorogenic action of epinephrine was still present in these animals, though it was slightly less than in frogs with the liver intact.⁷⁸

Phosphates.—The inorganic phosphate of blood plasma and the excretion of phosphate in the urine are temporarily diminished during epinephrine action.⁷⁹ Part of the muscle glycogen broken down under the influence of epinephrine does not reach the lactic acid stage but remains as hexosemonophosphate in muscle (Fig. 42). The formation of hexosephosphate occurs at the expense of the inorganic phosphate of muscle, and this leads to a fall in the concentration of plasma phosphate.

Insulin injections also cause a rise in hexosephosphate in muscle, which, since it does not occur if hypoglycemia is prevented by glucose injection or if the adrenals are removed, must be due to a reflex discharge of epinephrine elicited by the hypoglycemia. The decrease in plasma phosphate observed after insulin injections is only partly accounted for by the increase in hexosephosphate in muscle. Insulin plus glucose injected into adrenalectomized animals produces a fall in

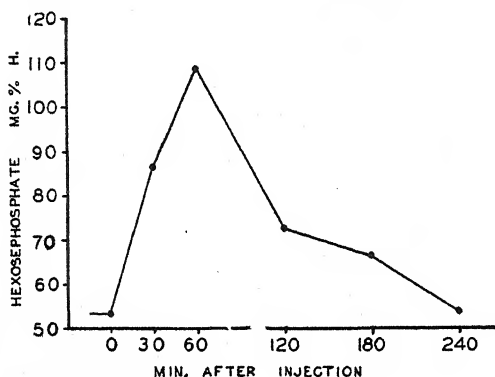


Fig. 42.—Effect of a subcutaneous injection of epinephrine (0.02 mg. per 100 Gm. rat) on hexosephosphate content (expressed as hexose) of muscle. (Data from Cori and Cori.⁴¹)

plasma phosphate in the absence of any hexosephosphate changes in muscle. Apparently, inorganic phosphate is used in the formation of an as yet unknown organic phosphate compound in the tissues.⁸⁰ When glucose is injected into a normal animal, there occurs a decrease in plasma and urine phosphate. This is apparently due to insulin secretion, since in depancreatized dogs the plasma phosphate remains unchanged when glucose is injected.⁸¹

Blood Lactic Acid.—Lactic acid is a normal constituent of mammalian blood. In resting human subjects, the blood contains 8 to 15 mg. per cent of lactic acid and in dogs, rabbits, rats and cats, similar values have been established. In man, moderate exercise, such as walking at a comfortable rate, does not increase the lactic acid level in the femoral vein.⁸² More strenuous exercise causes some rise; and after short and exhaustive work such as sprinting, the lactic acid may rise to 100 mg. per cent and more. In rabbits, moderate struggling

increases the lactic acid in the blood. The fact that this is accompanied by a rise in blood sugar makes it very probable that it is not only the struggling, but epinephrine secretion which is responsible for the increase in blood lactic acid. Elevated blood lactic acid has been found in various diseases in men, such as uncompensated heart disease, extensive carcinomatosis, pneumonia and chronic nephritis.

According to the present view, resting and even moderately exercised muscles do not add lactic acid to the blood. The red corpuscles, however, have been shown to change glucose to lactic acid (glycolyze). This process takes place anaerobically as well as in fully oxygenated blood. The rate of glycolysis varies in different species; it is ten times higher in rabbit than in sheep blood. The following order was found: Rabbit > guinea-pig > dog > cat > monkey > man > ox > pig > sheep.⁸³ It is assumed that the normal lactic acid content of the blood has its origin in the glycolytic activity of the erythrocytes. The muscles are, however, usually the source for any increase in blood lactic acid above the normal level.

Heart.—Apart from the liver, the chief organ concerned with the removal of blood lactic acid is the heart. This has only been recognized recently. It had been known for a long time that blood which circulates through a surviving mammalian heart gradually loses its glucose content; and Starling and others have determined the amount of glucose used per gram heart per hour under various experimental conditions. Starling⁸⁴ found that the heart of a diabetic dog uses less glucose than that of a normal dog—an observation which has been confirmed repeatedly. Addition of insulin accelerates the disappearance of glucose, and increases the glycogen content of a normal and raises the respiratory quotient of a diabetic heart.⁸⁵

The blood which feeds a surviving heart loses glucose not only because the heart takes it up, but also because the corpuscles split glucose to lactic acid. In previous experiments, this source of error was not taken sufficiently into consideration; *i. e.*, the total amount of glucose which disappeared from the blood was assumed to have been used by the heart, while actually a considerable amount of the glucose which disappears is changed to lactic acid by the corpuscles. Simultaneous determination of the lactic acid and sugar content in the arterial and coronary venous blood shows that the heart withdraws nearly ten times as much lactic acid as glucose from the blood.⁸⁶ Lactic acid must therefore be regarded as an important fuel of the heart. According to McGinty 40 per cent of the total O₂ consumption of the heart is accounted for by the lactic acid uptake. When surviving hearts are asphyxiated, the flow of lactic acid is reversed, the heart adds lactic acid to, instead of removing it from the blood. The source of this lactic acid is the heart glycogen, because this is found to diminish during asphyxia. Epinephrine has apparently no effect on heart glycogen.⁸⁷

During starvation the glycogen content of the heart rises progres-

sively, while that of skeletal muscle falls. One finds, in general, that the function of the heart and of other vital organs is better protected during starvation than that of less vital organs. Another interesting observation is that the heart in depancreatized dogs contains more glycogen than is found in normal hearts.

Nervous Tissue.—Brain seems to derive most of its energy from the oxidation of carbohydrate. The respiratory quotient of the brain *in situ*, determined by analyzing the arterial and venous blood for O_2 and CO_2 , was found to be close to unity under all conditions, even in diabetic animals.⁸⁸ Brain forms lactic acid from glucose but not from fructose, though it can oxidize the latter sugar.⁸⁹ Iodoacetate inhibits lactic acid formation and O_2 consumption of brain, while addition of lactate restores the O_2 consumption of brain tissue poisoned with iodoacetate.⁹⁰ This suggests that lactic acid is oxidized by brain. Since brain contains very little glycogen, it depends chiefly on blood sugar for the formation of lactic acid.

Lack of glucose in the blood causes first a stimulation of certain areas of the brain resulting in clonic convulsions (this may be similar to the initial stimulation of the brain seen in the case of anoxemia). If the lack of glucose in the blood persists for any length of time, a paralysis of the central nervous system follows, death resulting from a stoppage of respiration. The almost instantaneous restorative effect of an intravenous injection of glucose and the specific effect of this sugar, suggest that it is actually the lack of glucose in the blood which causes the symptoms. Narcotics depress the O_2 consumption of brain tissue.⁹¹

The respiratory quotient of peripheral nerve is close to 0.8, indicating that nerve, in contrast to brain, oxidizes chiefly noncarbohydrate material.⁹² Anaerobically, frog nerve forms lactic acid; after five to six hours, the rate of lactic acid formation falls off because the carbohydrate reserve from which lactic acid is formed approaches exhaustion, but when glucose is added to the Ringer's solution in which the nerve is suspended, lactic acid formation continues at a constant rate for twenty-four hours.⁹³ When O_2 is readmitted, part of the lactic acid which accumulated as the result of anoxemia, disappears again.⁹⁴ Electric stimulation does not accelerate lactic acid formation in nerve.

Glycolysis in Normal and Tumor Tissue.—Most tissues, when kept outside the body and deprived of O_2 , form lactic acid from glucose; or, in other words, they glycolyze. The highest rate of glycolysis has been observed in malignant tissue, in normal retina and in embryonic tissues; then, in descending order, brain > testicle = thymus > pancreas = liver = submaxillary gland = kidney > thyroid. When these tissues are kept in blood plasma and are fully oxygenated, only tumor tissue forms large amounts of lactic acid. This distinguishes tumor from normal tissues.⁹⁵ The distinction is, however, not absolute, since there are normal tissues which glycolyze in spite of full

oxygenation, such as mammalian erythrocytes. The lack of a nucleus, its small respiration and its comparatively short life span distinguishes the mammalian red cell from other cells. Nucleated bird erythrocytes do not glycolyze aerobically. Certain pathologic overgrowths, such as hyperplastic lymph nodes and inflammatory granulations caused by tubercle bacilli or by filtrable viruses,⁹⁶ also show aerobic glycolysis. Benign tumors have less aerobic glycolysis than malignant tumors.⁹⁵

Pasteur discovered that fermentation diminishes when yeast or other micro-organisms are brought from anaerobic into aerobic conditions. This effect of respiration on fermentation—the so-called “Pasteur reaction”—is also seen in the case of glycolyzing tissues and may be expressed as follows:

$$\frac{\text{Anaerobic-aerobic glycolysis.}}{\text{O}_2 \text{ consumption}}$$

Meyerhof calculates the quotient:

$$\frac{\text{Lactic acid disappearing in O}_2}{\text{Lactic acid oxidized}}$$

This so-called “oxidative quotient” was found to be between 3 and 6 for all tissues examined, including malignant tissue. The quotient indicates how many moles of lactic acid are removed (or are prevented from being formed) when one mole of lactic acid is oxidized. It will depend on the magnitude of anaerobic glycolysis in comparison to the magnitude of respiration, whether or not glycolysis persists when the tissues are supplied with oxygen. In malignant tissue, respiration has the usual diminishing effect on glycolysis, but since anaerobic lactic acid formation is very large in tumor tissue and O₂ consumption is not correspondingly increased, there remains some lactic acid formation in the presence of oxygen. In most normal tissues, with their comparatively low anaerobic glycolysis, respiration suffices to prevent lactic acid formation. If, however, normal tissues are subjected to procedures which decrease their respiration, lactic acid formation continues even in O₂; *e. g.*, rat placenta or spleen glycolyze aerobically when suspended in Ringer’s solution, but do not glycolyze in blood plasma. Ethyl cyanide is a chemical which in appropriate concentration neither influences respiration nor anaerobic glycolysis, but it inhibits the “Pasteur reaction” (*i. e.*, the effect of respiration on fermentation) so that anaerobic and aerobic glycolysis are of equal magnitude.⁹⁷

Glycolysis is an exothermic process and the energy liberated is, perhaps, used by the cancer cells for growth and multiplication. Tumor cells possess this source of energy over and above the energy which they derive from oxidations. The glycolytic activity has also been demonstrated for the tumor in the living animal, by means of analyses of in- and outgoing blood of the tumor for lactic acid.⁹⁸ The tumor was found to add lactic acid to the blood stream, while normal tissues do not contribute lactic acid to the blood if they are fully oxygenated.

The respiratory quotient of malignant tissue is below unity, in

contrast to other tissues with high anaerobic glycolysis (brain, embryo), which show a quotient close to unity.⁹⁹

Hyperinsulinism.—Many disturbances in carbohydrate metabolism are the result of abnormal insulin secretion. In recent times an increasing number of cases of hyperinsulinism have come under observation. These patients show severe symptoms of hypoglycemia when in a fasting condition and the blood sugar often drops to such low values that convulsions supervene. The blood sugar curve, after administration of glucose, remains abnormally flat and is followed by a pronounced hypoglycemic phase, a gross exaggeration of a type of blood sugar curve seen in certain normal individuals with marked postprandial hypoglycemia. Presumably, there are all transitions between these two types. In other cases of hyperinsulinism, one finds first a diabetic type of blood sugar curve, which is then followed by a precipitous fall in blood sugar to hypoglycemic values. Apparently, there is at first a sluggish response on the part of the pancreas, but when the blood sugar has reached sufficiently high values, an excessive insulin secretion sets in. Biopsies have revealed adenomas of the islands of Langerhans in a number of cases of hyperinsulinism and surgical removal of these adenomas has completely cured this condition.¹⁰⁰

More frequent are conditions associated with a hypofunction of the pancreas.

Starvation Diabetes.—When glucose is fed after a prolonged period of starvation, the blood sugar rises to very high values and a large part of the sugar fed is excreted in the urine. Owing to the long period of inactivity, insulin secretion of the pancreas is set in motion rather slowly. When a second dose of glucose is given a few hours later, it is utilized in a normal manner; *i. e.*, the pancreas has regained its functional activity under the influence of the first stimulus. The pancreas retains its ability of quick response for some time if starvation is again resumed; but eventually this faculty is lost again and the phenomenon described above may then be evoked a second time.¹⁰¹

Phlorhizin.—Phlorhizin injections produce a change in pancreatic function similar to that observed during starvation. If glucose is given to a phlorhizinized animal, the sugar is almost quantitatively excreted in the urine, but a second dose of glucose given a few hours later leads to a retention of part of the sugar.¹⁰² The insulin content of the pancreas of starved and of phlorhizinized animals was found to be approximately normal; so that the lack of response of the pancreas to the first dose of glucose would be due to a disturbance in secretion rather than production of insulin. When a small dose of insulin is injected into a phlorhizinized animal, there occurs immediately a marked decrease in sugar excretion in the urine; *i. e.*, the tissues are stimulated by insulin to an increased utilization of carbohydrate and they appropriate a certain amount of blood sugar for their own use, which would otherwise be excreted in the urine.¹⁰³

After removal of the kidneys, injection of phlorhizin has no effect on carbohydrate metabolism, which indicates that the changes observed in animals with intact kidneys are all secondary to the loss of sugar in the urine.¹⁰⁴

The following changes take place in the starving phlorhizinized animal. The continuous loss of sugar in the urine requires an increased production of blood sugar in the liver in order to prevent the blood sugar from falling. For a time the demand for blood sugar can be met from glycogen reserves present in the liver, but these soon become exhausted and the organism is forced to form blood sugar from non-carbohydrate sources. Now, if conversion of fatty acids to glucose were the principal mechanism of gluconeogenesis, one would expect an increase in the catabolism of fat at this stage; but what is actually observed is an increase in protein metabolism. The nitrogen excretion in the urine of starved and phlorhizinized animals is generally two to three times higher than that of starved normal animals.

As stated previously, not much carbohydrate is needed during simple starvation, and hence the protein metabolism remains low until the end, when a premortal rise in nitrogen excretion is observed. During phlorhizin poisoning, the demand for carbohydrate is unusually large and the increased protein metabolism, which is a terminal affair in simple starvation, occurs at an early stage. We might say, then, that phlorhizin poisoning is an exaggerated form of starvation. The phlorhizinized animal behaves as if there were a want of carbohydrate, while it is actually forming carbohydrate at the maximum possible rate, most of the carbohydrate formed being excreted in the urine.

As long as a certain minimum of carbohydrate oxidation persists, protein metabolism is held in check and the fatty acids can undergo complete oxidation. During phlorhizin poisoning the respiratory quotient is very low (0.69 to 0.71) and ketonuria is usually present. When the rate of carbohydrate oxidation is increased in a phlorhizinized animal, either by repeated administration of glucose or by injection of insulin, one finds a decrease in nitrogen excretion in the urine and a disappearance of ketonuria.¹⁰² When the effect of insulin wears off, protein metabolism rises again and incomplete oxidation of fatty acids reappears. This nitrogen-sparing and ketolytic effect of carbohydrate can also be demonstrated under other experimental conditions; it indicates that important interrelations exist between the metabolisms of carbohydrate, protein and fat.

The faculty of an animal to increase its protein metabolism for the purpose of new formation of carbohydrate, seems to depend on pituitary and thyroid activity. Phlorhizin injections in thyroidectomized or hypophysectomized animals produce less glycosuria than in normal animals, owing to the fact that the usual rise in protein metabolism is absent or does not occur to the same extent as in normal animals.¹⁰⁵

Since after depletion of the glycogen stores, protein is the main

source of the sugar excreted by the fasting phlorhizinized animal, the amount of nitrogen and sugar excreted in the urine should show a quantitative relationship. This is actually the case. The dextrose/nitrogen or D/N ratio in phlorhizinized dogs, cats, rabbits, goats and other species is generally in the neighborhood of 2.8.*

In a fasted animal kept under the action of phlorhizin the D/N ratio remains fairly constant from day to day and it is possible, therefore, to know how much glucose is formed from the endogenous protein metabolism. After feeding a single dose of glucose, 90 per cent or more of the sugar fed is excreted in the urine. It seems justified to assume that sugar formed in intermediary metabolism will have the same fate as that administered from without; *i. e.*, it will appear in the urine. If administration of a substance causes the excretion of an amount of glucose greater than that corresponding to a D/N ratio of 2.8, this is called "extra sugar" and is accepted as proof of the convertibility of this substance to glucose. One precaution is necessary in such experiments. Epinephrine injections cause an excretion of extra sugar in the urine of phlorhizinized animals. In this case the extra sugar comes from muscle glycogen which is broken down to lactic acid, the latter being converted to glucose in the liver. Conditions which lead to a breakdown of muscle glycogen must, therefore, be completely avoided in experiments designed to test the convertibility of a substance to glucose in the phlorhizinized animal.

When sugars other than glucose are fed to phlorhizinized animals, they are converted to a large extent to glucose before they are excreted in the urine. Besides, a great variety of noncarbohydrate substances (methylglyoxal, lactic, pyruvic, propionic, glyceric and succinic acid, glycerol, certain amino acids, to name only the most important ones) yield extra sugar in the phlorhizinized animal.¹⁰⁶ In so far as they have been tested, the same substances cause glycogen deposition in the liver of normal animals.

It may be calculated that a D/N ratio of 2.8 in a fasted, phlorhizinized animal corresponds to a conversion of 45 per cent of the catabolized protein to glucose. If meat is fed to a phlorhizinized dog, the sugar and nitrogen excretion rise, but the D/N ratio remains unchanged. The reason why only 45 per cent of the meat protein is converted to sugar is revealed in experiments in which the individual amino acids are fed to phlorhizinized dogs. One finds that certain amino acids are converted to glucose while others are not. To the former group belong all 3-, 4- and 5-carbon amino acids, except valine, which has a branched chain. Arginine is the only amino acid with more than 5 carbons, which is convertible to glucose, but the glucose is formed from the ornithine (5 carbon atoms), derived from the arginine. All straight-chain amino acids investigated, except lysine, yield sugar. Proline is the only cyclic amino acid convertible to glu-

* This applies to phlorhizin as now available on the market. For an unknown reason, samples of phlorhizin used up to 1926 gave a D/N ratio of 3.6 in dogs.

cose. Leucine, tyrosine and phenylalanine yield β -hydroxybutyric acid; *i. e.*, after deamination their fate is the same as that of higher fatty acids.¹⁰⁷

From the known facts regarding the convertibility of amino acids into glucose, and from the amounts of different amino acids in meat protein, it is possible to calculate a theoretical *D/N* ratio for this protein. Such a calculation shows that 100 Gm. of meat protein should yield 44 Gm. of glucose, corresponding to a *D/N* ratio of 2.7; this is very close to the average *D/N* ratio actually observed when meat is fed to phlorhizinized animals.

If one deducts the basal nitrogen and sugar excretion from that found after feeding a given amino acid, the proportion of the amino acid carbon converted to glucose may be calculated. It was found that all the carbon atoms contained in glycine, alanine, serine and cystine enter into the formation of glucose. The following amino acids yield 3 carbon atoms which enter into the formation of glucose: The 4-carbon dicarboxylic aspartic acid, which is supposed to be broken down by way of lactic acid; the 5-carbon dicarboxylic glutamic acid, which is supposed to be broken down to glyceric and acetic acid; and the 6-carbon amino-acid arginine which yields ornithine, which, in turn, may yield either succinic or glutamic acid. It would appear, then, that only such amino acids as contain, or can break up into, odd-numbered carbon chains can yield glucose, while even-numbered carbon fragments follow the same path in metabolism as the fatty acids.*

With few exceptions, naturally occurring fatty acids are even-numbered and break up into even-numbered carbon chains through the process of β -oxidation; when they are fed to phlorhizinized animals, they are not converted to glucose. Acetic acid, which is the first product of β -oxidation, likewise fails to yield extra sugar.¹⁰⁹ These results offer a strong argument against the idea that fatty acids are a source of sugar in the body.

Pancreatic Diabetes.—The most important disturbance in carbohydrate metabolism is that caused by the removal of the pancreas in animals and by pathologic lesions of the islet tissue of the pancreas in man.

Complete removal of the pancreas leads to the death of the animal in a short time, unless insulin is injected daily. Until recently, it was thought that all the symptoms of diabetes may be explained by the lack of insulin, but this supposition was considerably shaken by the observation of Houssay¹¹⁰ that hypophysectomized dogs do not develop severe diabetes when the pancreas is removed and that they survive for as long as six months. Adrenalectomized dogs also survive pancreatectomy for several weeks without insulin treatment.^{110a} The

* Glycine, which contains 2 carbon atoms, seems to be an exception to this rule. One may picture its conversion to glucose by way of glycol aldehyde ($\text{CH}_2\text{OH}\cdot\text{CHO}$). This two-carbon sugar polymerizes readily in aqueous solution to hexose and leads to the excretion of extra glucose when injected into the phlorhizinized animal.¹⁰⁸

amelioration of diabetes following hypophysectomy may be due to the fact that new formation of carbohydrate is disturbed in these animals. The significant fact that hypophysectomized dogs do not show the usual rise in protein metabolism, when injected with phlorhizin, has already been mentioned.

The metabolic disturbances produced by pancreatectomy are similar to those observed in phlorhizin poisoning. In the absence of food, endogenous protein is the chief source of urinary sugar and the D/N ratio is found to be in the neighborhood of 2.8 in the dog and cat, while supposedly complete human diabetics show a D/N ratio of about 3.6. The same substances which give rise to excretion of extra sugar in phlorhizinized animals, so far as they have been tested, do so likewise in depancreatized animals. There is no preferential utilization of galactose, fructose or dihydroxyacetone over glucose in diabetic dogs.¹¹¹

The respiratory quotient of severe human diabetics and of depancreatized animals has been determined frequently. Values between 0.68 and 0.72 are found in the majority of instances, though occasionally values considerably below 0.7 have been reported in the case of humans.¹¹² Ingestion of carbohydrate does not raise the quotient of depancreatized dogs¹¹³ and the ingested sugar is excreted almost quantitatively in the urine. Exercise leads to lactic acid formation in diabetic muscle,¹¹⁴ but the quotient does not rise during the period of activity.¹¹⁵ This is explained by the fact that the energy for the reconversion of lactic acid to muscle glycogen is derived from fat oxidation. It is difficult to see, however, why diabetic muscle should not be able to oxidize lactic acid, since insulin is apparently not concerned with its oxidation.

The theoretical respiratory quotient for meat protein is 0.8 in a normal animal. In a diabetic animal, the respiratory quotient for meat protein must be lower, because part of the carbon contained in protein, instead of being oxidized to CO_2 and given off in the lungs, is excreted as sugar in the urine. Assuming a D/N ratio of 2.7, corresponding to a yield of 44 per cent of glucose, the theoretical respiratory quotient for meat protein in a diabetic animal would be 0.73. In diabetic dogs fed large amounts of meat, so that the metabolism was carried on almost entirely at the expense of protein, an average respiratory quotient of 0.72 was observed, which is additional proof that the sugar appearing in the urine was derived from protein.¹¹⁶ If the sugar formed from protein had been oxidized, the quotient should have been in the neighborhood of 0.8, as was actually the case in normal dogs fed large amounts of meat. Apart from incomplete oxidation of protein, the respiratory quotient of diabetic animals may also be slightly depressed by incomplete oxidation of fatty acids; *i. e.*, by excretion of acetone bodies in the urine.

One group of physiologists holds that the chief disturbance in diabetes is not in the sugar-utilizing mechanism, but consists in an overproduction of sugar from fatty acids, so that more sugar is formed

than the animal can use.¹¹⁷ Hence, if glucose is given to a diabetic animal, it is excreted in the urine not because the animal is unable to use it, but because the animal is already using the maximal possible amount of sugar. When it is pointed out that feeding of fatty acids does not increase the sugar excretion of diabetic animals, while protein does, they reply that the animals are already forming the maximal possible amount of sugar from fatty acids, while they still retain the power of forming sugar from protein.

The respiratory quotient, according to the overproduction school, does not indicate the proportion of fat and carbohydrate that is undergoing oxidation, but is merely the algebraic sum of a fat-to-sugar transformation in the liver with a quotient of 0.3, and oxidation of the carbohydrate so formed in muscle with a quotient of 1. The fact that the quotient of the diabetic animal is rarely below 0.68 they attribute to a perfect coordination between the sugar formation from fatty acids and the oxidation of the sugar so formed. Obviously, if sugar formed from fat were temporarily stored as glycogen, the respiratory quotient would approach that of the hypothetical fat-to-sugar transformation, which is 0.3. Insulin, according to this view, does not stimulate oxidation of carbohydrate but merely suppresses the excessive formation of sugar from fat, the result being an increase in the respiratory quotient.

A necessary corollary of the overproduction theory is the assumption that the fuel of muscle is always carbohydrate. However, the respiratory quotient of muscle removed from diabetic animals was found to be that of fat.⁴⁴ Evidence of the same nature has also been obtained on nondiabetic animals; *i. e.*, if the respiratory quotient of the whole animal was that of fat, the quotient obtained on isolated muscle of the same animal was also that of fat.¹¹⁸ In hepatectomized rabbits, respiratory quotients of 0.75 to 0.8 have been observed.¹¹⁹ If muscle can burn fat directly, the fat-to-sugar transformation in the liver, with a transport of this sugar by way of the blood stream to provide fuel for muscle, becomes an unnecessary assumption.

Benign Glycosurias.—In some human subjects sugar appears in the urine after a glucose meal, though the blood sugar rises only to ordinary heights. The kidney threshold is lower than normal in these cases, and the condition is described as "renal diabetes," though the utilization of carbohydrate is normal. Pentosuria is a rare disease, of which, up to 1922, 35 cases had been reported. There occurs a continuous excretion of pentoses even during starvation and the sugar appears to be *L*-xyloketose.¹²⁰ Cases have been described which excrete fructose, often in considerable amounts, after ingestion of fructose or saccharose. During lactation, the urine often contains lactose.

Abnormal Glycogen Storage.—A most interesting syndrome has recently been described in children, consisting of a greatly enlarged liver, spontaneous hypoglycemia and ketonuria.¹²¹ The liver contains

abnormally large amounts of glycogen (up to 25 per cent) and is apparently unable to convert glycogen to glucose at a sufficiently rapid rate to keep the blood sugar at a normal level.

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REFERENCES

1. Cori, C. F.: *J. Biol. Chem.*, **66**, 691 (1925); *Proc. Soc. Exp. Biol. Med.*, **23**, 290; **24**, 125 (1926).
2. Trimble, H. C., Carey, B. W., and Maddock, S. J.: *J. Biol. Chem.*, **100**, 125 (1933); **107**, 133 (1934).
3. Wilbrandt, W., and Laszt, L.: *Biochem. Z.*, **259**, 398 (1933).
4. Lundsgaard, E.: *Biochem. Z.*, **264**, 209 (1933).
5. Woodyatt, R. T., Sansum, W. D., and Wilder, R. M.: *J. Am. Med. Assoc.*, **65**, 2067 (1915).
6. Maddock, S. J., Trimble, H. C., and Carey, B. W.: *J. Biol. Chem.*, **103**, 285 (1933).
7. Carpenter, T. M.: *Proc. Nat. Acad. Sci.*, **12**, 415 (1926).
8. Somogyi, M.: *J. Biol. Chem.*, **92**, xxii (1931).
9. Somogyi, M.: *J. Biol. Chem.*, **78**, 117 (1928); **103**, 665 (1933); Svedberg, A.: *Skand. Arch. Physiol.*, **66**, 113 (1933).
10. Trimble, H. C., and Carey, B. W.: *J. Biol. Chem.*, **90**, 655 (1931); Cori, G. T., Closs, J. O., and Cori, C. F.: *J. Biol. Chem.*, **103**, 13 (1933).
11. Folin, O., Trimble, H. C., and Newman, L. H.: *J. Biol. Chem.*, **75**, 263 (1928).
12. Foster, G. L.: *J. Biol. Chem.*, **55**, 291 (1923); Cori, C. F., and Cori, G. T., *Am. J. Physiol.*, **71**, 688, (1925); Himsworth, H. P., *Clin. Sci.*, **1**, 1 (1933).
13. Cori, C. F.: *J. Biol. Chem.*, **70**, 577 (1926).
14. Miller, M. M., and Lewis, H. B.: *J. Biol. Chem.*, **98**, 133 (1932); Silberman, A. K., and Lewis, H. B.: *J. Biol. Chem.*, **101**, 741 (1933).
15. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **76**, 755 (1928).
16. Stöhr, R.: *Z. physiol. Chem.*, **206**, 15, 211 (1932).
17. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **81**, 389 (1929).
18. Wilson, R. H., and Lewis, H. B.: *J. Biol. Chem.*, **85**, 559 (1930).
19. Catron, L. F., and Lewis, H. B.: *J. Biol. Chem.*, **84**, 553 (1929).
20. Eckstein, H. C.: *Proc. Soc. Exp. Biol. Med.*, **29**, 160 (1931-32).
21. Davenport, H. A.: *J. Biol. Chem.*, **70**, 625 (1926).
22. Somogyi, M.: *J. Biol. Chem.*, **105**, LXXXI (1934).
23. Lohmann, K.: *Biochem. Z.*, **178**, 444 (1926); Barbour, A. D.: *J. Biol. Chem.*, **85**, 29 (1929).
24. Browne, J. S. L., and Grant, R.: *Biochem. Z.*, **264**, 163 (1933).
25. Lesser, E. J.: *Biochem. Z.*, **119**, 108 (1921); **140**, 439; **156**, 161 (1923).
26. Lesser, E. J.: *Biochem. Z.*, **102**, 304 (1920).
27. Jarisch, A.: *Arch. ges. Physiol.*, **158**, 478 (1914).
28. Cannon, W. B., McIver, M. A., and Bliss, S. W.: *Am. J. Physiol.*, **69**, 46 (1924).
29. Major, S. G., and Mann, F. C.: *Am. J. Physiol.*, **102**, 409 (1932); Bodo, R. C., Co Tui, F., and Farber, L.: *Am. J. Physiol.*, **103**, 18 (1933).
30. Banting, F. G., and Others: *Trans. Roy. Soc. (Canada)*, Sect. V, **16**, 39 (1922).
31. Lesser, E. J.: *Biochem. Z.*, **55**, 355 (1914); Issekutz, B. von: *Biochem. Z.*, **147**, 246 (1924); **183**, 283 (1927).
32. Macleod, J. J. R., Noble, E. C., and O'Brien, M. K.: *Trans. Roy. Soc. (Canada)*, Sect. V, **18**, 129 (1924).
33. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **74**, 473 (1927).
34. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **70**, 557 (1926).
35. Best, C. H., Dale, H. H., Hoet, J. P., and Marks, H. D.: *Proc. Roy. Soc.*, **100B**, 55 (1926).
36. Lukens, F. D. W.: *Ann. Internal Med.*, **8**, 727 (1934).
37. Meyerhof, O.: *Biochem. Z.*, **178**, 395, 462 (1926); **183**, 176 (1927); Meyerhof, O., and Lohmann, K.: *Biochem. Z.*, **185**, 113 (1927).
38. Embden, G., and Zimmermann, M.: *Z. physiol. Chem.*, **167**, 114 (1927).
39. Lesser, E. J.: *Biochem. Z.*, **102**, 304 (1920).

40. Hegnauer, A. H., and Cori, G. T.: *J. Biol. Chem.*, **105**, 691 (1934).
41. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **94**, 581 (1931).
42. Mason, E. H.: *J. Clin. Investig.*, **2**, 521 (1926); Deuel, H. J. Jr.: *J. Biol. Chem.*, **75**, 388 (1927); Carpenter, T. H., and Lee, R. C.: *Am. J. Physiol.*, **102**, 635 (1932).
43. Campbell, W. R., and Maltby, E. J.: *J. Clin. Investig.*, **6**, 303 (1928).
44. Richardson, H. B., Shorr, E., and Loebel, R. O.: *J. Biol. Chem.*, **86**, 551 (1930).
45. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **79**, 343 (1928).
46. Colwell A. R.: *Am. J. Physiol.*, **91**, 679; **92**, 555 (1930); Cori, G. T.: *Am. J. Physiol.*, **95**, 285 (1930).
47. Meyerhof, O., and Boyland, E.: *Biochem. Z.*, **237**, 406 (1931).
48. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **84**, 683 (1929).
49. Meyerhof, O.: *Biochem. Z.*, **183**, 176 (1927).
50. Meyerhof, O., and Lohmann, K.: *Biochem. Z.*, **185**, 113 (1927); Warburg, O., and Christian, W.: *Biochem. Z.*, **242**, 206 (1931).
51. Morgan, W. T. J., and Robison, R.: *Biochem. J.*, **22**, 1270 (1928); Levene, P. A., and Raymond, A. L.: *J. Biol. Chem.*, **80**, 633 (1928).
52. Shaffer, P. A., and Friedemann, T. E.: *J. Biol. Chem.*, **86**, 345 (1930).
53. Ellis, N. R., and Hankins, O.: *J. Biol. Chem.*, **66**, 101 (1925).
54. Bleibtreu, M.: *Arch. ges. Physiol.*, **85**, 345 (1901).
55. Wierzuchofski, M., and Ling, S. M.: *J. Biol. Chem.*, **64**, 697 (1925).
56. Murlin, J. R.: *J. Gen. Physiol.*, **17**, 282 (1933); Stiles, W., and Leach, W.: *Proc. Roy. Soc.*, **113B**, 405 (1933).
57. Page, I. H., and Young, F. G.: *Biochem. J.*, **26**, 1528 (1932).
58. West, E. S., and Peterson, V. L.: *Biochem. J.*, **26**, 1720 (1932).
59. Höber, R.: *Arch. ges. Physiol.*, **233**, 181 (1933).
60. Rapport, D., and Ralli, E. P.: *Am. J. Physiol.*, **83**, 450 (1928).
61. Staub, H.: *Z. Klin. Med.*, **104**, 587 (1926).
62. Gayet, R., and Guillaumie, M.: *Comptes rend. soc. biol.*, **97**, 1613 (1927); **112**, 1194, 1327 (1933).
63. La Barre, J.: *Arch. Internat. Physiol.*, **29**, 227 (1927).
64. Jordan, E. M.: *Am. J. Physiol.*, **80**, 441 (1927).
65. Takane, R.: *Biochem. Z.*, **171**, 403 (1926).
66. Best, C. H., Hoet, J. R., and Marks, H. P.: *Proc. Roy. Soc.*, **100B**, 32 (1926).
67. Huxley, J. S., and Fulton, J. F.: *Nature*, **113**, 234 (1924).
68. Freudenberg, K., et. al.: *Z. physiol. Chem.*, **187**, 89 (1930); **213**, 248 (1932).
69. Mann, F. C., and Magath, T. B.: *Am. J. Physiol.*, **65**, 403 (1923).
70. Drabkin, D. L., and Ravdin, I. S.: *J. Biol. Chem.*, **87**, i (1930).
71. Herring, P. T., Irvine, J. C., and Macleod, J. J. R.: *Biochem. J.*, **18**, 1023 (1924); Voegtlin, C., Dunn, E. R., and Thompson, J. W.: *Am. J. Physiol.*, **71**, 574 (1925); Winter, I. B.: *Biochem. J.*, **20**, 668 (1926).
72. Cannon, W. B., and Rapport, D.: *Am. J. Physiol.*, **58**, 308 (1922-23).
73. Cori, C. F., Cori, G. T., and Buchwald, K. W.: *Am. J. Physiol.*, **93**, 273; **95**, 71 (1930).
74. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **79**, 309 (1928); **86**, 375 (1930).
75. Sahyun, M., and Luck, J. M.: *J. Biol. Chem.*, **85**, 1 (1929).
76. Boothby, W. M., and Sandiford, I.: *Am. J. Physiol.*, **66**, 93 (1923).
77. Corkill, A. B., and Marks, H. P.: *J. Physiol.*, **70**, 67 (1930).
78. Buchwald, K. W., and Cori, C. F.: *J. Biol. Chem.*, **92**, 355 (1931).
79. Perlzweig, W. A., Latham, E., and Keefer, C. S.: *Proc. Soc. Exp. Biol. Med.*, **21**, 33 (1923).
80. Cori, C. F., and Cori, G. T.: *Arch. Exp. Path. Pharm.*, **172**, 249 (1933).
81. Markowitz, J.: *Am. J. Physiol.*, **76**, 525 (1926).
82. Cook, L. C., and Hurst, R. H.: *J. Physiol.*, **79**, 443 (1933).
83. Somogyi, M.: *J. Biol. Chem.*, **103**, 665 (1933).
84. Knowlton, F. P., and Starling, E. H.: *J. Physiol.*, **45**, 146 (1912).
85. Cruickshank, E. W. H., and Startup, C. W.: *J. Physiol.*, **77**, 365 (1933); **81**, 153 (1934).
86. McGinty, D. A., and Miller, A. T. Jr.: *Am. J. Physiol.*, **103**, 712 (1933); Evans, C. L., et al.: *J. Physiol.*, **80**, 21 (1933).
87. Evans, G. T.: *J. Physiol.*, **82**, 468 (1934).
88. Himwich, H. E., and Nahum, L. H.: *Proc. Soc. Exp. Biol. Med.*, **26**, 496 (1929); *Am. J. Physiol.*, **101**, 446 (1932).

89. Loebel, R. O.: *Biochem. Z.*, **161**, 219 (1925).
90. Krebs, H. A.: *Biochem. Z.*, **234**, 278 (1931).
91. Quastel, J. H., and Wheatley, A. H. M.: *Proc. Roy. Soc.*, **112B**, 60 (1932).
92. Meyerhof, O., and Schmitt, F. O.: *Biochem. Z.*, **208**, 445 (1929).
93. Gerard, R. W., and Meyerhof, O.: *Biochem. Z.*, **191**, 125 (1927).
94. Schmitt, F. O., and Cori, C. F.: *Am. J. Physiol.*, **106**, 339 (1933).
95. Warburg, O., Posener, K., and Negelein, E.: *Biochem. Z.*, **152**, 309 (1924); Warburg, O.: *Biochem. Z.*, **204**, 482 (1929).
96. Crabtree, H. G.: *Biochem. J.*, **22**, 1289 (1928).
97. Warburg, O.: *Biochem. Z.*, **172**, 432 (1926).
98. Cori, C. F., and Cori, G. T.: *J. Biol. Chem.*, **65**, 397 (1925); Warburg, O., Wind, F., and Negelein, E.: *Klin. Wochschr.*, **5**, 829 (1926).
99. Dickens, F., and Simer, F.: *Biochem. J.*, **24**, 1301 (1930).
100. Howland, G., Campbell, W. R., and Maltby, E. J.: *J. Am. Med. Ass.*, **93**, 674 (1929); Graham, E. A., and Womack, N. A.: *Surg., Gynec., Obst.*, **56**, 728 (1933); Tedstrom, M. K.: *Ann. Int. Med.*, **7**, 1013 (1934).
101. Dann, M., and Chambers, W. H.: *J. Biol. Chem.*, **89**, 675 (1930); **95**, 413 (1932); **100**, 493 (1933).
102. Wierzechowski, M.: *J. Biol. Chem.*, **68**, 385 (1926); **73**, 417 (1927).
103. Cori, C. F.: *J. Pharmacol.*, **23**, 99 (1924).
104. Deuel, H. J., Jr., Milhorat, A. T., and Wilson, H.: *J. Biol. Chem.*, **74**, 265 (1927).
105. Dann, M., Chambers, W. H., and Lusk, G.: *J. Biol. Chem.*, **94**, 511 (1931-32); Biasotti, D., and Houssay, B. A.: *J. Physiol.*, **77**, 81 (1932).
106. Mandel, A. R., and Lusk, G.: *Am. J. Physiol.*, **16**, 129 (1906); Dakin, H. D., and Dudley, H. W.: *J. Biol. Chem.*, **15**, 127 (1913); Dakin, H. D., and Janney, N. W.: *J. Biol. Chem.*, **15**, 177 (1913); Greenwald, I.: *J. Biol. Chem.*, **16**, 375 (1913-14); Ringer, A. I., and Frankel, E. M.: *J. Biol. Chem.*, **18**, 233 (1914); Wood-yatt, R. T.: *J. Biol. Chem.*, **20**, xxii (1915); Chambers, W. H., and Deuel, H. J. Jr.: *J. Biol. Chem.*, **65**, 21 (1925).
107. Dakin, H. D.: *J. Biol. Chem.*, **14**, 321 (1913); Ringer, A. I., and Lusk, G.: *Z. Physiol.*, **66**, 106 (1910).
108. Sansum, W. D., and Woodyatt, R. T.: *J. Biol. Chem.*, **17**, 521 (1914).
109. Milhorat, A. T., and Deuel, H. J., Jr.: *Proc. Soc. Exp. Biol. Med.*, **24**, 667 (1926-27).
110. Houssay, B. A., and Biasotti, A.: *Arch. Pharmacodyn. Therap.*, **38**, 250 (1930); *Arch. Physiol.*, **227**, 664 (1931).
- 110a. Long, C. N. H., and Lukens, F. D. W.: *Proc. Soc. Exp. Biol. Med.*, **32**, 392 (1934-35).
111. Campbell, W. R., and Markovitz, J.: *J. Clin. Investig.*, **4**, 37 (1927); Bollman, J. L., and Mann, F. C.: *Am. J. Physiol.*, **107**, 183 (1934).
112. Joslin, E. P.: *Carnegie Inst. Washington*, Publ. 323 (1923).
113. Verzar, E.: *Biochem. Z.*, **44**, 201 (1912); Rabinowitch, I. M.: *J. Clin. Investig.*, **2**, 143 (1925-26).
114. Weber, C. J., Briggs, A. P., and Doisy, E. A.: *J. Biol. Chem.*, **66**, 653 (1925).
115. Chambers, W. H., et al.: *J. Biol. Chem.*, **97**, 525 (1932); Canzanelli, A., and Kozody, M.: *Am. J. Physiol.*, **103**, 298 (1933).
116. Ralli, E. P., Canzanelli, A., and Rapport, D.: *Am. J. Physiol.*, **96**, 331 (1931).
117. Macleod, J. J. R.: *The Fuel of Life* (Princeton, 1928); Soskin, S.: *J. Nutrit.*, **3**, 99 (1930).
118. Himwich, H. E., and Castle, W. B.: *Am. J. Physiol.*, **83**, 92 (1925).
119. Drury, D. R., and McMaster, P. D.: *J. Exper. Med.*, **49**, 765 (1929).
120. Greenwald, I.: *J. Biol. Chem.*, **89**, 501 (1930).
121. Van Creveld, S.: *Klin. Wochschr.*, **12**, 529 (1932).

Summarizing Articles

- Cori, C. F.: *Physiol. Rev.*, **11**, 143 (1931); *Annual Rev. Biochem.*, **2**, 129 (1933); **3**, 151 (1934); **4**, (1935).
- Dann, M.: *Yale J. Biol. Med.*, **5**, 359 (1933).
- Lusk, G.: *The Science of Nutrition* (Philadelphia, 1928).
- Macleod, J. J. R.: *Carbohydrate Metabolism and Insulin* (London, 1926); *Bull Johns Hopk. Hosp.*, **54**, 79 (1934).

- Mann F. C.: *Medicine*, 6, 419 (1927).
Meyerhof, O.: *Die chemischen Vorgänge im Muskel* (Berlin, 1930).
Nash, T. P., Jr.: *Physiol. Rev.*, 7, 385 (1927).
Peters, J. D., and Van Slyke, D. D.: *Quantitative Clinical Chemistry*, vol. i, Chapter II (Baltimore, 1931).
Rapport, D.: *Physiol. Rev.*, 10, 349 (1930).
Richardson, H. B.: *Physiol. Rev.*, 9, 61 (1929).
Shaffer, P. A.: *Physiol. Rev.*, 3, 394 (1923).
Shaffer, P. A., and Ronzoni, E.: *Annual Rev. Biochem.*, 1, 247 (1932).
Trendelenburg, P.: *Die Hormone* (Berlin, 1929).
Warburg, O.: *Über den Stoffwechsel der Tumoren* (Berlin, 1926).

CHAPTER XXI

LIPID METABOLISM

THE LIPIDS

OTHER terms—The Fats, The Fatty Substances, Fats and Lipoids, Lipins.

Substances having the following characteristics:

- (a) Insolubility in water and solubility in the fat solvents, such as ether, chloroform, benzene.
- (b) Fatty acids, or related to the fatty acids as esters, either actual or potential.
- (c) Utilization by living organisms.

CLASSIFICATION

Simple lipids. Esters of the fatty acids with various alcohols.

Fats—esters of the fatty acids with glycerol.

Waxes—esters of the fatty acids with alcohols other than glycerol.

Compound lipids. Esters of the fatty acids containing groups in addition to an alcohol and fatty acids.

Phospholipids—substituted fats containing phosphoric acid and nitrogen—lecithin, cephalin, sphingomyelin.

Glycolipids—compounds of the fatty acids with a carbohydrate and containing nitrogen but no phosphoric acid—cerobrosides.

Aminolipids, sulfolipids, etc.—groups most of which are at present not sufficiently well characterized for classification.

Derived lipids. Substances derived from the above groups.

Fatty acids of various series.

Sterols—mostly large molecular alcohols, found in nature combined with the fatty acids and which are soluble in the fat solvents—cholesterol ($C_{27}H_{45}OH$), myricil alcohol ($C_{30}-H_{61}OH$), cetyl alcohol ($C_{16}H_{33}OH$), etc.

*Almost all the known lipids are found in living organisms, so that the general characteristics of the group are in the main those of naturally occurring substances. But such substances as have been produced synthetically behave, as far as is known, like the natural ones, and there is no reason to believe that the characteristics of the group will have to be altered to suit synthetic members. Thus this classification which was intended for biochemical purposes answers very well in the wider sense as a chemical classification.

The most general characteristic of the group is the solubility in fat solvents such as ether, chloroform, benzene, as contrasted with the insolubility in water. This of itself is sufficient to set it off from the

* The first part of this chapter is intimately bound up with Chapters III and IV.

other great groups of biological substances—the carbohydrates, proteins, and mineral salts. The property is not absolute, since certain members of the group, such as the lecithins, form dispersions on mixing with water, which are at least colloidal and may approach true solubility. On the other hand, many members of the group are not soluble in all fat solvents. For example, the phospholipids are insoluble in acetone, cephalin is insoluble in alcohol, while sphingomyelin and the cerebrosides are difficultly soluble in ether.

In order to exclude organic compounds which have no biochemical relationship to the fats or fatty acids, but which from their solubilities alone would be included in the group, the limitations in (b) and (c) have been applied. The substances included in the group must be either ester-like combinations of the fatty acids or capable of forming such combinations, and they must be capable of performing some useful function in living organisms.

THE SIMPLE LIPIDS

Fats are esters of the fatty acids with glycerol. These are commonly called *oils* when they remain liquid at ordinary temperatures, and *fats* when solid. They are the most important of the lipids from the point of view of quantity, wideness of distribution, food value, and commercial interest. They constitute the main form of food storage in animals, and share with carbohydrates and, to a less extent, with proteins this function in plants. As they occur naturally they are always mixtures of triglycerides of various fatty acids, and their properties vary with the nature of the fatty acids in the separate glycerides, and with the nature of the glycerides composing the mixture.

The glycerides of the higher fatty acids are insoluble in water, those of the lower fatty acids, *e. g.*, butyric, are slightly soluble. In the organic solvents such as ether, chloroform, benzene, all are readily soluble even in the cold and much more soluble hot. In ethyl and methyl alcohol and acetone they are slightly soluble in the cold but readily soluble when hot. In fact, boiling ethyl alcohol is one of the best solvents for use in extracting tissues, giving a cleaner extraction than ether, chloroform or benzene; probably because, owing to its affinity for water, it penetrates the tissue better. The solubility in alcohol, like the melting point, varies with the nature of the combined fatty acid, the glycerides of the unsaturated and the lower fatty acids being more soluble than those of the higher and saturated acids. The glycerides of the hydroxy fatty acids like the acids themselves are insoluble in petroleum ether.

Melting Point and Solidifying Point.—In general, the melting points of the glycerides are higher than those of the contained fatty acids, and vary with the fatty acids: The glycerides of the higher saturated acids having the highest melting points, those of the lower fatty acids lower, and those of the unsaturated acids still lower. The melting point of a natural fat, which is always a mixture of glycerides,

depends on the nature of the component glycerides. Its melting point may be low because it contains either glycerides of the lower acids or glycerides of the unsaturated acids. The melting points of mixtures of pure glycerides cannot be foretold from the melting points of the constituents. Eutectic mixtures are formed of which the melting points pass through a characteristic minimum value below that of either of the constituents. On the other hand, having determined the curve of melting points of various known mixtures of pure triglycerides, it is possible to determine the composition of an unknown mixture with a fair degree of accuracy, a fact which has been made use of by Twitchell.¹⁶⁷

The solidifying point of a glyceride or mixture of glycerides is always lower than the melting point, the difference being generally considerable and often wide. Thus, the melting point of tristearin is given as 71.5° , its solidifying point 52.5° .¹¹³ A sample of beef fat (from the heart) melted at 49.5° and solidified at 36° .¹ Grün and Schacht⁶⁵ synthesized three mixed glycerides which could be prepared in either the lower (labile) or higher (stable) melting forms. The labile form could be gradually converted into the stable by seeding with a crystal of the stable form, but the reverse change was not possible. The results of these workers seem to indicate that some unknown factor comes into play, possibly the existence, as suggested, of two forms of the glycerides; although from our present knowledge of the structure of the glycerides, it is difficult to see how such forms could be explained. From a practical point of view, the rule has originated that to get a true melting point it is necessary for the fat to stand at least twenty-four hours in the melting-point tube before the determination is made.

The delayed solidification appears to be of considerable importance in the living animal, since many stored fats have a melting point considerably above body temperature; while the solidifying point is some degrees below (note beef fat above).*

Color, Odor, etc.—The pure triglycerides are colorless, odorless and tasteless; the properties of color, odor and taste when present being due entirely to foreign substances mixed with or dissolved in them. Thus, the desirable yellow color of butter is due to plant pigments carried over from the food. Plant pigment is also responsible for most of the color of the stored fat of animals.^{137, 138} The flavor of food fats is also due to foreign materials absorbed by the fat, either from its natural environment, or formed during the processes of preparation. In modern butter making, the bacterial flora is carefully controlled with this point in mind.

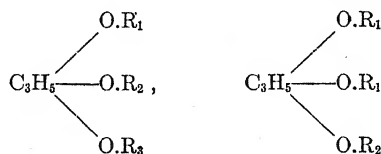
Glycerides.—The glycerides composing the natural fats may be either simple, containing only one fatty acid, or mixed, containing more than one acid; and, far from being rare constituents of the natural

* A condition has been noted in infants (*sclerema neonatorum*) in which the subcutaneous fat has hardened resulting in the death of the infant.¹⁶⁷ In this case the abnormality found was a high content of free fatty acid.

fats, as was first believed, more recent investigations tend to show that mixed glycerides form the bulk of many of the natural fats. For example Hilditch and his associates,^{13, 77} as the result of extensive studies on the make-up of the natural stored fats of plants and animals, have found that there is, generally, a definite pattern in the distribution of the fatty acids between simple and mixed glycerides, which is characteristic of the different groups.

Mixed glycerides have been prepared synthetically. The methods of synthesis are not markedly different from those for the simple glycerides, but the procedure is somewhat complicated by the fact that there is more or less shifting of the radicals in the molecule. For example, Kreis and Hafner⁹⁶ found that when oleic acid was allowed to act on dipalmitin and distearin, considerable quantities of tripalmitin and tristearin were found, while the yields of oleodipalmitin and -stearin were correspondingly reduced. It should be noted that the phospholipids, lecithin and cephalin are naturally occurring mixed triglycerides, containing, generally, two different fatty acid radicals and one phosphoric acid radical.

Optical Properties.—Certain of the mixed triglycerides should be optically active, since they contain an asymmetric carbon atom. Thus:



should be optically active.

As a matter of fact, the only optically active fats which are found in nature are those which contain optically active fatty acids; as, for example, castor oil and chaulmoogra oil; and those which contain optically active nonfat substances, such as resins, sterols (cholesterol, phytosterol, etc.). Unsuccessful attempts have been made, from time to time, to prepare optically active glycerides of the higher fatty acids.^{2, 15}

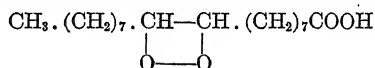
Hydrolysis (Saponification).—Fats are hydrolyzed in the laboratory in the same way and with the same agents as are simple esters. Water at high temperature (under pressure) as in the autoclave may be used either alone, or, more advantageously, with catalysts such as acids or alkalis. At ordinary pressures the same catalysts do the work, but more slowly. The rate of reaction may be increased by the use of a solvent, such as alcohol, which dissolves the fats. Amyl alcohol is more effective than ethyl alcohol, probably because of the higher temperature which can be obtained.

Enzyme hydrolysis is most effective when the fats are emulsified. Fat-splitting enzymes or lipase are present in the gastro-intestinal secretions of animals, and in many plants, especially in fatty seeds as the castor bean, seeds of *chelidonium majus*, the rubber plant, etc.

Synthesis.—Synthesis of the glycerides is carried out along the same lines as synthesis of the simple esters, but is complicated by the fact that glycerol is a triatomic alcohol with two different positions, and by the fact that there may be shifting of the groups from one position to the other. The special problems involved are reviewed by Amberger and Bromig,⁴ and the question of optical activity of the glycerides by Bergmann and Sabetay¹⁵ and Abderhalden.²

Synthesis of fats by enzymes has been successfully demonstrated with castor bean lipase by Welter,¹⁷⁰ and Armstrong and Gosney,⁹ who gave a beautiful demonstration of the reversibility of the synthetic-hydrolytic powers of the castor bean lipase. Synthesis by lipase of the pancreatic juice has been demonstrated by Hamsik,⁶⁶ Taylor¹⁶² and Foa.⁵¹

Rancidity.—Dry air, in darkness, has apparently no effect on oils and fats. Air in presence of moisture, and particularly of light and heat, rapidly brings about these changes known collectively as rancidity. Unsaturated fats become rancid more quickly than saturated, and free fatty acid formation appears to be the first essential for rancidity. The stages in the process have been outlined by Kerr and Sorber⁹¹ as follows: first the development of free acid, then a drop in free acid coincident with the rancidity, followed again by increased acidity. The iodine value falls, unsaponifiable matter increases, and oxygen is fixed in peroxide form of the following nature:



This substance then acts as a carrier of oxygen for the production of various oxidation compounds, the nature of which is unknown. Greenbank and Holm⁵⁹ found that metals catalyze powerfully the production of rancidity.

Waxes—*esters of the fatty acids with alcohols other than glycerol* are very widely distributed in the plant and animal kingdom, their main usefulness being apparently as protective agents, due to their chemical inertness. The high lipid content of the tubercle bacillus is due mainly to the waxes and wax alcohols it contains.⁵ Insect waxes and leaf waxes are esters of higher alcohol and mainly higher saturated acids. Thus, the main constituent of beeswax is myricyl palmitate.

A group of waxes which is of particular importance to the biochemist is that of the esters of cholesterol and related sterols with various fatty acids. In animals these occur in largest amounts in blood plasma. Similar esters may occur in plant tissues, but so far, no mention has been made of them. The cholesterol esters of blood plasma have been found to consist mainly of esters of palmitic, oleic and linolic acids with smaller amounts of stearic and other acids. In addition to the blood plasma, the esters are to be found in large amounts only in the suprarenal glands. Small amounts occur in the liver, kidney, heart, and probably in other organs and tissues, but the

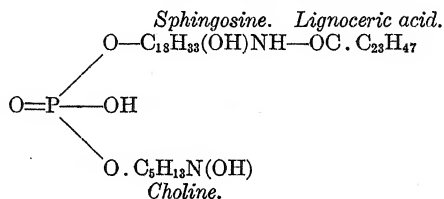
quantities are generally so small that there is always a question whether they are real constituents of the tissues or are due to the blood plasma present. In some abnormal conditions, such as amyloid kidney, and the fatty liver produced by feeding cholesterol, liver, etc., undoubted deposits of esters occur.^{16, 17, 34} The waxes are considerably more difficult to hydrolyze than the fats, and this difference has been made use of in separating them; in particular, cholesterol esters, from the fats.¹⁶⁵ For complete saponification of the cholesterol esters, special means must often be employed; for instance, the use of sodium ethylate on the esters in ethereal solution.⁵⁶

COMPOUND LIPIDS

Phospholipids.—We are indebted largely to MacLean in England and to Levene in this country for clearing up this previously very complicated field, and reducing the large number of poorly determined and doubtful compounds to a few which they have been mainly instrumental in defining. The best characterized members of the group are the lecithins, cephalins, and sphingomyelin.

Sphingomyelin.—The substance yields, on hydrolysis, at least three fatty acids—stearic ($C_{18}H_{36}O_2$), lignoceric ($C_{24}H_{48}O_2$), and nervonic ($C_{24}H_{46}O_2$); two bases—sphingosine and neurine or choline; and phosphoric acid.

A formula suggested by Levene¹⁰⁴ is as follows:



Properties.—Sphingomyelin is a relatively stable substance, undergoing no change in air or light, is soluble in hot alcohol (from which it separates on cooling in crystalline form), relatively insoluble in cold or hot ether, and easily soluble in cold or hot chloroform, benzene, pyridine and glacial acetic acid. It is insoluble in cold, but somewhat soluble in hot acetone. It mixes with water to form an opalescent suspension from which it is precipitated by acetone. It is dextro-rotatory, having a specific rotation of about eight.

Sphingosine, the chief base, is an unsaturated amino alcohol, containing two hydroxyl groups, one primary amino group and one double bond, with the empirical formula of $C_{18}H_{37}NO_2$ and with the probable composition of $CH_3(CH_2)_{12}.CH=CH.CHOH.CHOH.CH_2.NH_2$. Sphingomyelin occurs in brain, kidney, liver, egg yolk, and in small amounts in blood and muscle.

Lecithin and Cephalin.—These substances contain the same elementary constituents—two molecules of fatty acid, one of phosphoric acid, one of glycerol and one of base. In lecithin the base is choline,

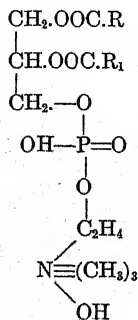
in cephalin it is aminoethyl alcohol. They are found associated with each other in all tissues. It should be added that while the structure of lecithin may be said to be known, that of cephalin is still in doubt, since analyses of the substance as prepared in supposedly pure form give values which do not agree with its formula.

The number of lecithins or cephalins is limited theoretically only by the number of fatty acids, since each new arrangement of fatty acids would mean a new compound. Practically, however, the number of different substances of this nature found in the animal body is probably limited to a few, although very little information is available on this point. The phospholipid of cellular material is probably to be regarded as essential to the life of the cell since it is preserved in constant proportion even in extreme emaciation.^{124, 146} Its exact function is still unknown; although, as indicated below, it may have to do with the metabolism of the fatty acids or possibly with the processes of oxidation in the cells.

Properties.—The lecithins and cephalins are soluble in the fat solvents with the single and characteristic exception of acetone. They are miscible with water forming cloudy solutions from which they may be precipitated by acetone. Ordinary lecithins and cephalins oxidize in air, turning brown and taking on a disagreeable odor. They have no definite melting point but decompose on heating. They combine with both acids and bases and form combinations with the most diverse substances—salts, proteins, carbohydrates—most of which are not to be considered as chemical compounds but rather adsorption mixtures; it is probable that lecithin exists in tissues in this type of combination. They undergo hydrolysis more readily than the fats.

In preparing them advantage is taken of these properties. Thus lecithin is separated along with cephalin from the other lipids by virtue of its insolubility in acetone. Lecithin is separated from cephalin by taking advantage of the insolubility of the latter in alcohol. The phospholipids are separated from water-soluble impurities by solution in water and precipitation with acetone; from organic impurities, by combination with cadmium chloride, etc.

Lecithin.—The structure of lecithin generally accepted is as follows:¹²¹



This is the asymmetrical form. A symmetrical form is possible, with the phosphoric acid radicals attached to the middle carbon of glycerol; but since all known lecithins are optically active, the asymmetrical formula is probably the correct one. The mode of attachment of the choline to the phosphoric acid is still a matter of dispute, although the ester form of combination as above is generally accepted.

Lecithin has been synthesized by different workers, Grün and Kade,⁶³ Grün and Limpacher⁶⁴ and most recently by Levene and Rolf.¹⁰⁸ Grün and Limpacher indicate another possibility in the lecithin formula—the possibility of an anhydride form by loss of water from the phosphoric acid and choline residues. Levene, Rolf and Simms¹¹¹ removed one of the fatty acids (the unsaturated acid) by the action of cobra venom on lecithin, and from the lysolecithin so formed built up other lecithins.

The differences between various lecithins are largely, if not altogether, due to the fatty acids which they contain; and these have as yet been insufficiently studied. As far as present information goes, most lecithins contain one saturated and one unsaturated fatty acid; although as Sinclair¹⁵⁵ has pointed out, and as indicated by the work of others in this laboratory, first, not more than 30 to 40 per cent of the phospholipid fatty acids have been found to be saturated; and second, if each phospholipid had one saturated and one unsaturated acid in the molecule, the iodine number of the unsaturated acids, calculated from the iodine number of the mixed acids, would be impossibly high. The inference is that there are some phospholipids containing only unsaturated acids. Levene and Simms¹¹⁰ found that the liver lecithins contained the saturated acids palmitic and stearic, and oleic and arachidonic as the unsaturated acids, although linolic acid was not excluded. Levene and Rolf¹⁰⁶ found that egg yolk contained oleic and small amounts of linolic and arachidonic acids. In a lecithin prepared from the soy bean, Levene and Rolf¹⁰⁹ found stearic and palmitic acids, and oleic, linolic and linoleic acids. The proportion of unsaturated acids was relatively low as compared with animal lecithins. As noted below the fatty acid composition of the animal lecithins may be altered by the fat of the food.

Cephalin.—The cephalins have been much less studied than the lecithins. They are distinguished from the lecithins by their insolubility in alcohol, by containing aminoethyl alcohol in place of choline, and possibly by a difference in molecular structure. Otherwise, their properties and behavior are much the same.

Like the lecithins, they are believed to contain one saturated and one unsaturated acid in each simple molecule, and of these, the saturated acid is apparently stearic. The unsaturated acids of brain cephalin were found by Levene and Rolf¹⁰⁷ to be oleic and arachidonic. MacArthur and Burton¹¹⁷ found that cephalin from sheep and beef brain contained fatty acid in the following approximate proportions—stearic acid 30 per cent, oleic acid 55 per cent, cephalinic acid 10 per

cent, and clupanodonic acid 5 per cent. However, as MacLean has pointed out, no one else has found as high a percentage of oleic acid in cephalin. Parnas¹³⁹ came to the conclusion that the only saturated acid of cephalin was stearic. Recent work by Klenk⁹² indicates that the cephalins contain largely stearic acid and C_{22} unsaturated acids, and that the lecithins contain palmitic acid and C_{20} unsaturated acids.

Galactolipids. Cerebrosides.—These substances, of which there are at least four, contain galactose, the base sphingosine, and a fatty acid, but no phosphoric acid. They differ from each other only in the nature of the fatty acid which they contain; *phrenosin* containing cerebronic acid ($C_{24}H_{48}O_3$), *cerasin* containing lignoceric acid ($C_{24}H_{48}O_2$), *nervone* containing nervonic acid ($C_{24}H_{46}O_2$) and *oxynervone* containing oxynervonic acid ($C_{24}H_{46}O_3$). In solubility, they closely resemble sphingomyelin, and the separation from this substance is difficult. They dissolve readily in hot alcohol, acetone, or benzene, but are almost insoluble in ether, hot or cold. They dissolve in pyridine at room temperature. They occur in largest amounts in the brain and nerves, and are of special interest to the biochemist because they contain galactose, which is not known to occur in any other combination in the tissues; and also because they contain a sugar and a fatty acid in the same molecule, which is of interest from the fact, noted below, that the fatty acids, in the later stages of their metabolism, seem to require the assistance of the carbohydrates for combustion. They make up a considerable proportion of the myelin sheath of nerves.

DERIVED LIPIDS

Fatty Acids.—The important series of fatty acids are:

(A) The saturated, straight-chain series, $C_nH_{2n}O_2$, including practically all the even-numbered carbon atom acids up to and including C_{30} . The more commonly occurring ones in the natural fats are palmitic (C_{16}) and stearic (C_{18}) acids which occur in practically all. Palmitic acid is the most widely distributed, and, quantitatively, is the most important. Of the others which are of notable importance to the biochemist may be mentioned lignoceric, $C_{24}H_{48}O_2$, found in sphingomyelin and cerasin, and butyric, $C_4H_8O_2$, in butter.

(B) The series with one double bond, $C_nH_{2n-2}O_2$, the oleic or acrylic series. The practically important member of this series is oleic acid, although several other members of the series are known; for example, hypogeic (C_{16}), gadoleic (C_{20}), erucic (C_{22}) together with isomers of each, having the double bond in different positions.

Oleic acid is the most widely distributed of all the acids of the fats, and is also, quantitatively, the most important. It is found in several isomeric forms but the commonly occurring one has the constitution $CH_3(CH_2)_7CH=CH.(CH_2)_7COOH$. Another oleic acid was found by Hartley,^{69, 70} among the fatty acids of liver, and has the double bond between the sixth and seventh carbons. It is interesting

to note that he also found in liver a C_{18} acid with two double bonds, one between the sixth and seventh, and one between the ninth and tenth positions, the occurrence of which would be most readily understood by supposing that the ordinary oleic acid of the food or stores had acquired a new linkage between the sixth and seventh carbons.

Acids more unsaturated than oleic acid are found in vegetable oils and in animal tissues. In the former, they are present as triglycerides (fats), while in animal tissues very little is present as fat, the larger part being as phospholipids. The presence of these more highly unsaturated acids in the vegetable oils gives them the important commercial property of "drying"; i. e., of forming, by oxidation, a water-proof skin or varnish over surfaces on which they are spread, as in painting. The unsaturated acids of animal tissues, although they oxidize in air in a similar way, become sticky instead of forming a smooth skin. These differences may probably be referred to differences either in the length of chain or position and number of the double bonds.

While oleic acid is quite stable in the presence of oxygen at ordinary or body temperatures, the more unsaturated acids take up oxygen and undergo other changes with a readiness dependent on the degree of unsaturation. Leathes has observed that it is rare to find fatty acids in the fat stores of the animal body more unsaturated than oleic acid, which he believes is due to the fact that oleic acid may be stored without oxidation at body temperature, while a more unsaturated fat cannot.

(C) *The Linolic Series*, $C_nH_{2n-4}O_2$. The known acids of this series are all C_{18} acids. They were studied first in linseed oil and have since been found to occur rather widely in animal tissues; for example, in pig liver,⁷⁰ blood,²⁵ egg yolk,¹⁰⁶ muscle, etc. Except in the liver, and then only in relatively small amount, they do not occur in ordinary stored fat of mammals, but are found mainly as phospholipids or as esters with cholesterol.

The structure of most of these acids is not known. They react with alkaline permanganate in the cold in a similar way to oleic acid, forming hydroxyacids, and on further oxidation yield short-chain acids.

Linolenic Series, $C_nH_{2n-6}O_2$. A C_{18} acid of this series, or rather two isomeric acids, have been prepared from linseed oil.⁴⁷ Some light on their constitution was obtained by decomposition of the ozonide which yielded sufficient azelaic acid to account for half the molecule. In addition, malonic acid and propionic aldehyde were obtained. Traces of an acid of which the bromine derivative is insoluble in ether but soluble in benzene have been found in blood plasma.

Linolenic acid has been reported among the unsaturated acids of brain by Grey.⁶¹

Other series. Of the acids of other series, very little of biochemical importance is known. Arachidonic acid, $C_{20}H_{32}O_2$, was found in pig liver,⁷⁰ and later in the lecithin from the same source.¹¹⁰ Amounts

generally less than 5 per cent of the total fatty acid, of acids giving bromine derivatives insoluble in ether and benzene have been found in the cholesterol ester fractions of blood plasma, and in the lecithin (acetone-insoluble) fractions.²⁷ This is probably a very low figure, because of the formation of isomeric compounds which are soluble in ether.

Levene has found arachidonic acid in brain cephalin and lecithin.¹⁰⁷

Solubility.—The lower members of the various fatty acid series up to C_6 are miscible with water in all proportions. Caproic acid (C_6) is soluble in water at 15° to the extent of about 0.9 per cent. The solubility decreases rapidly with increasing length of chain. All the saturated acids above lauric acid are insoluble in water. In hot absolute, or 95 per cent alcohol, all fatty acids are soluble, but the higher acids are all sparingly soluble in cold alcohol.

All fatty acids are soluble in ether, chloroform, benzene, etc. All except the hydroxy fatty acids are soluble in petroleum ether.

The hydroxyacids are more or less soluble in water depending on the number of hydroxyl groups; thus octahydroxy arachidic acid is easily soluble in water, hexahydroxystearic more difficultly soluble, while tetrahydroxystearic acid requires 2000 parts of boiling water for solution. The highly hydroxylated acids are insoluble in ether and difficultly soluble in alcohol. The dicarboxyacids are more soluble in water than the corresponding monocarboxyacids, and, in general, less soluble in the fat solvents. The fatty acids found in food fats are soluble in bile or bile salts; and recent work indicates the mechanism by which the solution of the fatty acids in bile salts is brought about.¹⁶⁹ The bile salts form combinations with the fatty acids which are soluble in water and diffusible, and these combinations represent the form in which the fatty acids and also cholesterol are absorbed.

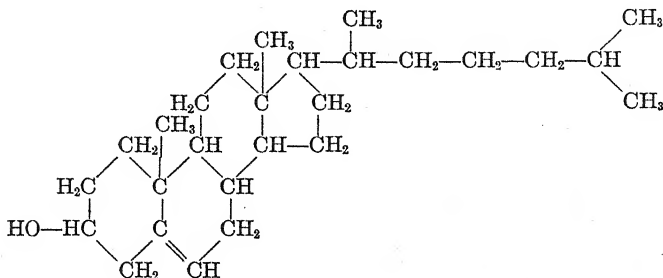
Hydrogenation.—The unsaturated acids take up hydrogen at the double bonds with the aid of catalysts and become saturated. Sabatier and his associates were the first to show that finely divided metals, and particularly nickel, were the best catalysts. Since that time the process has become of great commercial importance in changing comparatively inedible oils, such as cottonseed oil, into valuable articles of diet. As far as can be determined, the hydrogenated fats are just as well utilized by the animal body as the natural fats.⁹⁸ A review of the work on hydrogenation of oils has been published by Sabatier.¹⁴⁷

Halogen Absorption.—The halogens are readily absorbed at the double bonds, and under certain conditions the absorption is quantitative; so that the halogen absorption value (iodine number) constitutes one of the most important means of study of the fatty acids. The more unsaturated acids (three and four double bonds) form halogen absorption derivatives which are insoluble in most fat solvents, and this properly serves for their identification and approximate determination.

STEROLS

The alcohols belonging in this group are those found combined in the waxes, and of these the only ones directly connected with life processes are cholesterol in animals and various phytosterols in plants. Of these, cholesterol is the only well-known one.

Cholesterol.—Formula, $C_{27}H_{46}O$; constitution:¹⁷⁶



Cholesterol is insoluble in cold water, sparingly soluble in cold alcohol, readily soluble in hot alcohol and in fat solvents generally. Its melting point is about 148°C . It is levorotatory, the rotation varying somewhat with the solvent. For ether $\alpha_{\text{D}}^{15} = 31.1^{\circ}$.⁷⁶ On exposure to light and air, it slowly undergoes changes—its melting point is lowered, its solubility changed, and its color reactions rendered indefinite.

Its iodine absorption value varies with the solution used. With Hübls' reagent it gives practically theoretical values (67 to 68 as compared with 65.8, the theoretical value). Wijs' solution gives erratic results. The more recent Rosenmund-Kuhnhehn procedure also gives theoretical results.¹⁴⁴

Methods for Detection and Determination.—The methods which are most useful for small amounts of cholesterol are:

(A) *The Liebermann-Burchard method*, depending on the color developed in a chloroform solution of cholesterol by acetic anhydride and sulfuric acid. A fine green color is finally produced, which, under exactly defined conditions, is quantitative. Based on this reaction, a considerable number of methods for its determination in blood and other body fluids have been developed; but the fact that there are so many methods indicates that the determination by color production is not particularly satisfactory. Reasons for the difficulty have been indicated, among them the fact that rosin acids and terpenes give similar reactions,¹¹² that the tint and development of the color is influenced by light and temperature,²² and that the esters of cholesterol influence the tint.⁵⁷ Nevertheless, the colorimetric methods take relatively little time as compared with the precipitation method (noted below); and when conditions are carefully controlled, give results of sufficient accuracy for most purposes.

(B) *The Windaus Digitonin Precipitation*.—Cholesterol unites quantitatively with digitonin to form an addition product which is practically insoluble in alcohol or ether. The esters do not combine with digitonin; so that it is possible by this means to make a quantitative separation of free cholesterol from its esters.^{174, 175} This useful method in many modifications is being widely used especially on the micro scale. Although generally regarded as more accurate than the colorimetric method, it is not free from objection.¹⁴⁸

The gravimetric determination of cholesterol in tissues is beset with difficulties, owing to the fact that when separated in the ordinary way in the "unsaponifiable" fraction after hydrolysis of the tissue with alkali, it is contaminated with much other material from which it is difficult to separate it.

Cholesterol is found in the tissues of all animals examined.³⁹ In warm-blooded vertebrates, it is the only sterol found; but in lower animals and insects, somewhat variant forms are present. They all have the same formula and differ only in crystal form and in the melting points of their dibromides and acetates. The unsaturated linkage and the —OH which Hausmann⁷² found to be essential for its anti-toxic functions are found in all forms. Cholesterol never occurs in plants, and in animals appears to be formed almost exclusively by synthesis. It apparently cannot be formed from the plant sterols.

Isocholesterol differs from cholesterol in being dextrorotatory ($\alpha_D = +60^\circ$ in ether), and in having a lower melting point (136°). It is less soluble in cold alcohol than cholesterol and gives a yellow color with the Liebermann-Burchard reagent. It occurs in wool wax.

Phytosterols are closely related to the cholesterol in composition and have many similar properties. They hold a position in plants corresponding to cholesterol in animal tissues. Phytosterol palmitate has been found in corn pollen.⁶

The distribution of the sterols in various fats and oils has been reported by Stuart.¹⁶⁰

METHODS OF STUDY OF TISSUE LIPIDS

Two general procedures are available for removal of lipids from tissues and their later analysis:

Extraction.—Removal of the lipids from the tissues in approximately the form in which they exist there. In the experience of the writer, the solvent which gives most nearly complete extraction is boiling alcohol. It has two disadvantages—it extracts other substances than lipids, and the heat probably decomposes some of the more sensitive ones. Nevertheless, it is probably superior to all others for the purpose, since it can be applied directly to the tissue without previous drying with its almost inevitable oxidation; and because it penetrates the tissue readily, breaking up whatever loose combinations there may be between lipid and other cellular constituents. Cold alcohol is preferred by many because there is less danger of decompo-

sition of the lipids. It takes much longer than the hot solvent and gives a less complete extraction. Others of the fat solvents are used for special purposes; *e. g.*, acetone for dehydration and to remove cholesterol and free fat, leaving the phospholipids. Solvents like ether and chloroform extract very little from fresh tissues; and while they give better results with dried material, the extraction is probably never complete. Preliminary treatment of the tissue with alcohol or acetone results in a better extraction with these solvents. The purification of the individual lipids is often a matter of difficulty, and the procedures cannot be described in a brief review. The reader is therefore referred to special articles.^{26, 82, 105, 121, 166}

Hydrolysis—destroying the tissue by the use of strong alkali, then extracting the separated fatty material. This procedure is to be recommended when a quantitative measure of the total lipid content is required; but it has the disadvantage of giving very little information regarding the lipids as they are in the tissue. Two fractions are obtained—the fatty acids and the “unsaponifiable matter,” the latter consisting of the sterols and a number of unknown substances, part of which are probably related to them.¹⁰² The procedure was first made use of by Liebermann, and developed in detail by Kumagawa and Suto,⁹⁷ and later by Lemeland.¹⁰³

Methods for analytical examination and the determination of the physical and chemical characteristics (“constants”) of the fatty acids have been well worked out, but since they are given in all good treatises on the chemistry of the fats they will not be described here. Much attention has been given in recent years to the development of micro-methods for measurement of the lipids so that a satisfactory knowledge of the percentage composition may be obtained on a gram or two of tissue or tissue fluids, permitting study of the lipids without killing the animal.

PHYSIOLOGIC ASPECTS

This section will deal with the fats and related substances more particularly in their behavior in the animal organism.

Digestion.—Fat differs from the other foodstuffs in that it has very few forms which are water-soluble; and probably for this reason an entirely different mechanism is made use of in the animal body for its transport through the intestinal wall and in the blood and tissues.²⁴ It differs from other foodstuffs, first in that it is resynthesized before it leaves the intestine; and second, in that it is carried to the blood by a roundabout way; *i. e.*, *via* the lymph system and the thoracic duct, reaching the general circulation before it passes through the liver. When it finally reaches the liver, it does so by way of the arterial circulation.

Fat is carried in the blood as *fine* particles, while other digestion products are carried in solution. In its digestion it follows the general rule of breaking down into its constituent elements, glycerol and fatty

acids, just as the proteins are reduced to amino acids and the carbohydrates to monosaccharides; and while absorption in undigested form cannot at present be excluded, the facilities for digestion are ordinarily so adequate, that it is questionable whether any escapes hydrolysis. Enzymes which can accomplish the splitting (lipases) are to be found in the gastric and pancreatic secretions and, to a less extent, although still in notable amounts, in the secretions of the glands of the intestine itself. These lipases are all in water solution and can therefore act only on the surface of the fat particles; and splitting can take place to a notable extent only when the available surface of the fat is large—as when emulsified. The main emulsifying agent in the intestine is soap, formed by the union of the free fatty acid in the fat with the alkali of the bile and pancreatic secretions. The fatty acid present in small amounts in all food fats is increased by the processes of cooking and by the gastric lipase until when the fat reaches the intestine, it has enough free fatty acid in it to insure good emulsification. Mucin in the bile and other secretions, and lecithin in the bile act to increase the stability of the soap emulsion.

Ordinarily, there is little fat-splitting in the stomach; first, because a soap emulsion cannot form in an acid medium; and, second, because the gastric lipase is destroyed by acid. Appreciable hydrolysis takes place only when the fat is in emulsified form, as in milk; and when enough protein or other neutralizing agent is present to reduce the gastric acidity to a point where the lipase destruction is slow. Another condition under which fat-splitting may take place in the stomach is when for any reason (such as an excess of fat in the food) emptying of the stomach is delayed; then intestinal fluids containing bile and pancreatic secretions back up into the stomach.

In the intestine, the most important factor in digestion and absorption, not excepting the pancreatic secretion, appears to be the bile; since when it is missing, fat absorption falls to a lower level than when bile is present and pancreatic secretion is absent. The great importance of the bile rests in its power of dissolving soaps and free fatty acids in which solution they are readily absorbed. The important constituents of the bile which accomplish this end are the bile acids, glycocholic and taurocholic acids.¹³²

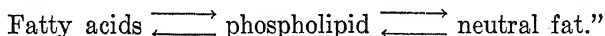
The absence of pancreatic lipase appears to have no great effect on the hydrolysis of the fat, since owing to the presence of lipases in the intestinal secretion this is practically complete, whether pancreatic lipase is present or not.¹³⁰ The reason why so much fat escapes absorption under these conditions is probably that hydrolysis is greatly delayed; and, owing to reabsorption of bile which is known to take place, there is no bile salt left in the intestine to aid in the passage of the fatty acids through the absorbing cells.

Absorption.—The way in which the fat passes through the absorbing surface of the intestine and reaches the thoracic duct is still not well understood. The first conception, the passage of unchanged fat

in finely particulate form, has been largely given up because of the facilities for complete splitting present in the intestine. The fat ordinarily enters the intestine in small amounts, and there is enough lipase present in the pancreatic and intestinal secretions to hydrolyze many times the amount ordinarily present. The next and, at present, prevailing conception is that the fat is completely hydrolyzed and absorbed either as fatty acids or soaps. The fact that the small intestine is faintly acid through most of its length is a difficulty in the way of assuming the presence of soaps. The emphasis in aiding fat absorption falls at present on the bile salts. The mode of action of these substances developed by Verzář and his associates¹⁰⁸ is an evolution from the "choleic acid principle" of Wieland, and consists in the formation of compounds of the bile salts with the fatty acids and cholesterol, which are water-soluble and diffusible, and which thus pass the epithelial walls. Since there is not enough bile salts secreted to combine with the fatty acids, there is assumed either circulation of the bile salts—absorption and resecretion—or a fixing of the bile salts in the walls of the absorbing surface where they allow or aid the passage of the fatty acids and cholesterol; which is Verzář's opinion.

The constituents of the fat, the glycerol and fatty acids, having been absorbed into the epithelial cells of the mucous membrane, are then recombined into fat and passed on into the thoracic duct in finely divided form. The details of this resynthesis are not known, but the observations of Sinclair¹⁵³ furnish the basis for a plausible hypothesis. He found that during fat absorption (a) the percentage amount of phospholipid in the intestinal mucosa does not change; but (b) the nature of a portion of the phospholipid, *i. e.*, the fatty acids contained in it, does change in accordance with the nature of the fatty acids being absorbed. His hypothesis is stated as follows:

"Within the epithelial cells of the intestinal mucosa there is a specific phospholipid which occupies an intermediary position between fatty acids and neutral fat, as represented by the equation:



When cholesterol is present, it is partly esterified during absorption. It is not absorbed in appreciable amounts unless fat is being absorbed at the same time. These facts are in line with the suggestion which has been made by Hanes,⁶⁷ in connection with fat and cholesterol ester formation from phospholipid in the developing chick, that two molecules of phospholipid provide fatty acids enough for one molecule of fat and one of cholesterol ester.

The resynthesized fat does not go directly into the blood stream but passes with the lymph stream through the thoracic lymph duct into the venous circulation. It is still an unsettled question as to whether any part of the fat is absorbed directly from the intestine into the

blood stream. Only about 60 per cent has been recovered from the thoracic duct where it empties into the circulation, which leaves 40 per cent to be accounted for. Attempts to demonstrate direct absorption of fat into the blood have not been given satisfactory results;^{48, 180} although this path of absorption is not by any means eliminated. Recent work goes to show, however, that there are pathways between the lymph and venous circulations other than the main opening of the thoracic duct—a finding which will go far to explain the discrepancy. Thus Lee,¹⁰¹ after ligation of the thoracic duct, found that connections were established between the lymphatic duct and the azygos vein or its branches, and also with the right thoracic duct. He gives also a good review of the literature showing that similar results have been reported previously, concluding that it is well established that the lymph, and therefore the absorbed fat, does not necessarily all enter the venous system at the base of the neck. These findings are borne out by the work of Eckstein.⁴¹

As regards other lipids—lecithin when fed has sometimes been found to give a slight increase in the blood lecithin⁴² and in the lecithin of the chyle.¹⁵⁶ More recent work has failed to support these findings⁴¹ and goes to show that lecithin, like the fats and simple esters, is probably hydrolyzed in the intestine and the resulting fatty acids built up into fats during absorption. Simple esters such as the ethyl esters of the ordinary fatty acids are hydrolyzed in the intestine and the fatty acids are built into glycerides, no evidence of the original ester or its contained alcohol being found in the chyle.^{19, 53, 136}

Modifications of Fat During Absorption.—The fat of the chyle is not always the same as the fat of the food. Frank⁵³ found after feeding ethyl stearate that the melting point of the fatty acids was much below that of either stearic acid or tristearin. Bloor,²⁰ after feeding olive oil, found that the fat of the chyle melted around 30°. These differences may mean either that the absorbed fat was diluted with body fat from some source, or that chemical changes take place—such as desaturation in the case of the stearic acid and saturation in case of oleic acid. Work by McClure and Huntsinger¹²⁰ shows that the fat in the blood after absorption need not be the fat of the food; and the work of Tangl and Berend,¹⁶¹ and Berend¹⁴ indicates a possible mechanism for desaturating the fatty acids during absorption.

After feeding coconut oil, Raper found that the fatty acids of chyle showed a higher average molecular weight than that of the oil fed; which he believed indicated that the lower acids of the oil probably reached the blood stream as soluble salts.¹⁴²

Digestibility and Absorbability of Fat.—There is a prevailing belief among dietitians that certain fats are more digestible than others, that the vegetable fats and the recently exploited hydrogenated fats are less well utilized by the animal body than the animal fats. In an investigation of this point, Langworthy⁹⁸ made use of 23 animal, 34 vegetable and 6 hydrogenated fats in feeding experiments on human

beings. The results showed very little difference in the behavior of the fats studied. In general, the utilization was found to be inversely proportional to the melting point, the high melting fats being more difficult of utilization. His results show that, aside from melting point, the origin of the fat is not of great importance to its utilization.

After long feeding, fat may be less well utilized than at first. Thus Pettenkofer and Voit¹⁴⁰ in a fifty-eight-day feeding period of 500 Gm. meat and 200 Gm. fat daily to a 32-kilo dog, found that the feces contained as high as 38 per cent of fat at the end of the period, while at the beginning, absorption was complete (98 per cent).

Parenteral Absorption.—Fat as such, or preferably as an emulsion injected under the skin, or into the abdominal cavity, is absorbed in considerable amounts. It passes into the lymph system and much of it enters the blood by the usual channel—the thoracic duct.^{129, 179}

Homogenized fat injected intravenously produces a sudden rise of blood fat of 100 per cent or more, then a slow fall. Not over 44 per cent of the injected fat was present in the blood at any time.¹⁴¹ Fat in coarser suspension appears to be treated like other suspended foreign matter—it is removed rapidly, the coarse particles in the lungs, the finer ones in the liver, spleen and bone marrow.

Absorption of Cholesterol.—Free cholesterol alone is absorbed but little if at all from the intestine. When dissolved in fat or fatty acid it is slowly absorbed with partial esterification. Mueller¹³⁵ found that when cholesterol was fed either free or as esters (together with fat) there was an increase of both in the chyle, the proportion between the two remaining approximately the same as is found normally in blood plasma; indicating that esterification or hydrolysis may take place in the intestinal walls. Hueck and Wacker⁸¹ found that both free and bound cholesterol could be increased in rabbits' blood by feeding cholesterol, but they could not get an increase in the blood of cats and dogs. Grigaut and L'Huilliere⁶² succeeded in producing these effects in dogs by feeding cholesterol. Knudson⁹⁴ found that during the absorption of olive oil there was a marked increase in cholesterol esters but no increase in total cholesterol in the blood. In later work⁹⁵ he fed pure cholesterol and cholesterol esters to dogs and found that both were absorbed; but in every case the increase was in the free cholesterol and not in the esters. These results are in disagreement with those of Gardner and coworkers,⁵⁴ who found that when cholesterol was fed to rabbits there resulted an increase in esterified as well as free cholesterol. Knudson believes that the exclusion of fat from his diets is responsible for the failure of the esters to increase. The part played by the bile salts in cholesterol absorption is shown by the work of Verzár.¹⁶⁸ In summing up, the absorption of cholesterol appears to require the simultaneous absorption of fat and the presence of bile salts. A considerable proportion is esterified during absorption.

Fat in the Blood.—The fat passes from the intestine into the thoracic duct and to the blood in the form of a very fine suspension which

persists for a few hours and then disappears. The fine particles of fat are about 1μ in size, are visible with the highest power of the microscope, and a count of the number in a given volume gives a measure of the rate at which fat is reaching the blood.⁵⁵ The measure is only approximate, since the number in the blood at any time is the resultant of the number entering and the number leaving the blood during the period of absorption. During the period in which absorbed fat is present in the blood, the lipid phosphorus of the blood increases.^{12, 21, 180} Cholesterol has been found to increase (generally late in the period of absorption) by some workers¹⁶³ but most others have not found any increase unless cholesterol was present in the fat fed.

The manner in which the fat leaves the blood is not known. During fat absorption it accumulates in the blood, in the liver, and to, a less extent, in other organs. Colored fat in fine emulsions injected into the circulation is found to collect in the liver, bone marrow, spleen and muscles in the order named; in which respect it behaves like other foreign material.²⁹

Since fat is hydrolyzed before passing the intestinal cells, the question has been raised whether a similar hydrolysis is necessary before it can pass into the tissue cells.¹¹⁴ Such a process would require the presence, in the blood or in the cells where fat was being deposited, of enzymes capable of splitting the fats. Such has been shown not to be the case and fat passing the tissue cell walls as such to and from the blood, probably does so without the aid of lipases.⁸¹

In this connection, confusion has arisen because of the universal presence of an enzyme which readily splits simple esters, such as ethyl butyrate and glycerides of the lower fatty acids, but acts only slowly on the glycerides of ordinary fat. This enzyme is called lipase by many workers. Since it also hydrolyzes lecithin and possibly cholesterol esters, it is probably of considerable importance; but enough evidence has accumulated to show that it is not a true lipase and should not be so called.¹⁷³ The name suggested by Loevenhart for this enzyme is *esterase* which seems to be a good one. While esterases are widely distributed in animal tissues⁴⁹ lipases appear to be confined to the pancreas and intestine.

LIPIDS IN THE TISSUES

Stored Fat.—It has long been known that fat was one of the forms in which energy is stored in plants and animals; and the nature of the fat and the way in which it was formed and laid down have been the object of scientific and commercial interest.

In animals, fat may occur in all organs and tissues; but the three most important places of permanent deposit are the intermuscular connective tissue, the abdominal cavity, and the subcutaneous connective tissues. In plants, the seeds and fruits and, in certain cases, the roots are rich in fat; and in winter, fat is to be found in the trunks of trees.

The nature of the stored fat in warm-blooded animals has been extensively studied. It consists, ordinarily, largely of a mixture (in varying proportions) of the glycerides of the three fatty acids, palmitic, stearic, and oleic acids. Esters of various other fatty acids, such as lauric, myristic, and linolic are sometimes present in small amounts and are probably to be referred to the food. In cold-blooded animals, the stored fat contains a larger proportion of the unsaturated acids than does the fat of warm-blooded animals. In plants there occurs the greatest variety of combined fatty acids, practically all the known even-numbered fatty acids occurring in combination in plant products.

Stored fat in animals originates in the food, representing either material synthesized from carbohydrate or protein, or transferred more or less directly from the fat in the intestine.

Each species of animal, under normal conditions, lays up a fat which is to a considerable extent characteristic of the animal; thus, it is not difficult for even the beginner to distinguish between lard and beef tallow; and the chemist has been able to work out a set of characteristics for each animal and vegetable fat by means of which he is able to distinguish one from the other with some degree of accuracy. In plants, there is only one possible source of the stored fat—carbohydrate; and as a consequence, the "constants" of vegetable fats are much more definite than those of animal fats. In animals, the fat of the food may, by forced feeding, be transferred to the fat stores with little if any change; but when the animal has a normal choice of food, the fat which is stored is generally quite different from that of the food. Modifications may be brought about in the fat absorbed in the following known ways: (a) by changes during absorption; (b) by choice as to which of the constituents of the food fat is burned and which stored; (c) by admixture with fat synthesized from carbohydrate. Very little is known about any of these methods of modification; so that their relative importance cannot be estimated. Regarding (a), although changes have been shown to take place in both hard and soft fats, the way in which the change is produced, whether by dilution with fat from bodily sources or by chemical change, is not known. Fat synthesized from carbohydrate is "hard" fat and the practice among hog raisers of "finishing" the animals by a period of grain feeding is to harden them; *i. e.*, to produce a firm fat. Scientific foundation for the practice has been furnished by the work of Anderson and Mendel,⁸ who found that with a preponderance of carbohydrate in the diet, a characteristic hard fat was produced; while with a preponderance of various oils, a fat corresponding to these oils could be stored. Anderson⁷ showed, in addition, that in adult animals which had been raised on various oils, the oily stored fat may be changed to a hard fat by feeding with starch. Jackson⁸⁵ lists the fats of ordinary feeds in the following order of their ability to produce a soft fat: most effective linseed, then soy bean, maize, beechnut, cottonseed, wheat, pea, oat, rice, peanut, barley, rye, and bean. Much significant

work on this topic has been done by Ellis and his associates.⁴⁴ The distribution of stored fat in animals and the effect on it of various factors have been extensively studied in Mendel's laboratory.¹⁴³

Shioji¹⁵² showed that the phospholipids of the tissues may also be somewhat changed by the fat of the food, but to a much less degree than the stored fat. Sinclair¹⁵⁴ has defined the conditions under which the fatty acids of the tissue lipids (phospholipids) may be altered by the fat of the food. In general, it was found that the degree of unsaturation of the fatty acids of the phospholipids may be readily increased but the lowering is a matter of greater difficulty. The phospholipids seem to absorb the unsaturated acids selectively.

Fat in the Liver.—The liver of animals ordinarily contains considerable amounts of carbohydrate and not much fat. The fat which it contains is, however, considerably more unsaturated than the fat of the stores. Under certain conditions (fasting, phosphorus poisoning in certain diseases, and to a certain extent after fat feeding) the fat content of the liver increases. Fat has been found to accumulate in the liver of young animals before birth. Imrie and Graham⁸⁴ studied the fat content of the livers of embryonic guinea-pigs throughout the period of gestation and found that, from the time, when the embryo is 35 to 40 Gm. in weight to the time of birth, the fat content increases from 2 to 3 per cent up to 16 to 18 per cent of the moist tissue, and has a comparatively high iodine value. After birth the fat diminishes rapidly in the first forty-eight to seventy-two hours.

Many reasons have been offered for the accumulation of fat in the liver. Of these, one of the earliest, that of fatty degeneration (the transformation of liver protein into fat) has been abandoned, since exact methods have provided no evidence to support it. Another was that of an antagonism between glycogen and fat. When the liver is full of glycogen, fat cannot be deposited there in notable amounts, but when the glycogen is exhausted or low, fat flows in to take its place.¹¹⁵ The flow of fat to the liver in diabetes and, in general, in carbohydrate starvation has been taken as support for the idea that fat is transformed to carbohydrate; which is believed to take place in diabetes.⁵⁸ It should be noted that carefully controlled work in this country gives little support to this view.¹¹⁵ In the writer's opinion, the most rational reason for the accumulation of fat in the liver is given by the Leathes hypothesis of fat metabolism, according to which the flow to the liver is a normal process; since the first stage of fat catabolism (desaturation and phosphorization) probably takes place there.

Biochemical Synthesis of Fatty Acids.—*From Carbohydrates.*—It has long been known, practically, that animals can synthesize fat from the carbohydrate of the food and the scientific proof has not been difficult to supply. In plants, formation from carbohydrate is the accepted method of fat formation. Nevertheless the details of this all-important synthesis are practically unknown.

Synthesis from Proteins.—It has been shown by feeding experiments with experimentally diabetic animals that about two thirds (58 per cent) of the protein molecule is convertible into carbohydrate.⁸⁶ Since carbohydrate is readily convertible into fat, it follows that protein should be also. The difficulty of demonstrating the conversion is great, owing to the fact that, because of the bulk of ordinary protein food and also because of its stimulating effect on metabolism, animals cannot readily be made to eat enough to provide a surplus.

In recent work Atkinson, Rapport, and Lusk¹⁰ report as follows: When the glycogen reservoirs are low, ingestion of large quantities of meat results in deposition of glycogen. Further meat feeding results in retention of both fat and glycogen; while on stuffing with meat, fat alone was formed. After a carbohydrate meal the evening before (glycogen stores full), ingestion of meat produces fat.

Tissue Lipids.—As has just been noted, there is always a large proportion of the fatty material of tissues which cannot be seen with the microscope, cannot be stained by the usual fat stains and cannot be extracted by the ordinary fat solvents such as ether, chloroform or benzene; but may be removed, although some of it with difficulty, by alcohol either cold or hot. The nature of this lipid material and its relationship to the fat of food and stores constitutes one of the important problems in the field of fat metabolism at the present time.

The most widely distributed of these constituents are the phospholipids of various composition which are present in practically all tissues and in blood. Their function is ordinarily assumed to be in connection with the intermediary metabolism of the fatty acids, since they carry in combination the more unsaturated acids found in the body. Cholesterol, either as such or as its numerous isomers, is also universally distributed; and, in the form of its esters, with various fatty acids is one of the main lipids of blood plasma. Cholesterol esters are found in tissues and organs, but only in such small amounts that their presence there may be accidental as the result of adherent blood plasma. Practically all the known lipids have been prepared from animal and plant tissues.

In addition to the lipids normally present in tissues, others may be deposited in abnormal conditions. Thus the "anisotropic" fat of the pathologists appear to consist, characteristically, of combinations of cholesterol with the fatty acids and may be produced by excessive feeding of cholesterol.^{11, 122} Under these circumstances, the deposits take place in the Kupffer cells of the liver and in the adrenals, corpus luteum, spleen and endothelium of the blood vessels. The suprarenal glands are characterized by the large amounts of cholesterol and cholesterol esters which they contain. Cholesterol crystals may be found in any tissue that is undergoing slow destruction, especially where absorption is poor, accumulating as one of the least soluble of the cell constituents. Feeding cholesterol, whole liver, or the alcoholic extract of liver results in accumulations of fat and cholesterol esters

in the liver.^{16, 17, 34} These accumulations may be prevented by feeding lecithin, choline or betaine. Other pathologic accumulations are being recognized and given special names; for example, *Gaucher's* disease in which there is a great accumulation of cerebrosides in the spleen, the *Schüller-Christian* disease with accumulations of phospholipid in the liver and various other places, and other conditions grouped under the name of xanthomatosis where the accumulations are cholesterol and cholesterol esters largely in the skin.¹⁴⁵

The lipid percentages of normal blood and tissues (other than fat) are characteristic of the tissue and remain relatively constant over a wide range of bodily conditions including starvation,^{125, 164} although with the progress of the latter some differences appear. This constancy of percentage of phospholipid and cholesterol, which persists over a wide range of nutritional conditions, even of fasting to death, indicates that these substances are true cellular constituents. Their content is highest in the brain, heart, and kidney, then in the muscles and lowest in the blood plasma. In muscle the content is highest in those muscles which are most used—heart, diaphragm, jaw, pectoral muscles of flying birds, etc. The part which they play in life processes is still to be discovered. The phospholipids and cholesterol appear to act as a balanced metabolic pair in many processes.³⁸ In severe diabetes and a few other conditions the percentages of blood lipids are often considerably above normal and in some cases very much above.^{18, 60}

THE OXIDATION OF THE FATS IN THE LIVING BODY (INTERMEDIARY METABOLISM)

The Glycerol Part of the Fat.—The available evidence indicates that glycerol is utilized in the same way as the carbohydrates. Thus, when fed to completely phloridzinized animals it is excreted as sugar;^{36, 115} and, in the consideration of the foodstuffs as to whether they produce or prevent the production of the acetone compounds, it is found to behave as an antiketogenic substance.¹⁵⁰

The Fatty Acids.—The manner of disposal of the long chains of the fatty acids offers greater difficulties. One of the suggested early stages in fat utilization is the formation from it of phospholipids by replacement of one of the fatty acid groups by a phosphoric acid complex—generally either phosphoric acid-choline as in the lecithins, or phosphoric acid-amino-ethyl alcohol, as in the cephalins. The evidence regarding the phospholipids as intermediary compounds in the utilization of the fats is, first, the universal presence of these compounds in living tissues and organs; second, the increase of lipid phosphorus in the blood and liver during fat absorption when the blood contains much extra fat (alimentary lipemia);^{12, 21} third, the increased values for lipid phosphorus in lipemia of other origins;²³ and fourth, evidence of formation of milk fat from phospholipid of blood. Thus, Meigs, Blatherwick and Cary,¹²⁷ were able to show as a result of the analysis of the blood before and after passing through the milk gland

of the cow, that the fat of milk originated mainly if not entirely in the phospholipid of the blood. In work reported at an earlier date Jordan, Hart and Patten found in cows that a diet low in phosphorus resulted in a lower milk fat production than when the phosphorus supply was adequate. The change affected mainly the glycerides of the volatile and soluble fatty acids—which would require more phosphorus for lecithin formation than the higher acids.⁸⁸ Lastly it has been possible to show that the phospholipids of the hen's egg may be influenced by the fat of the food¹¹⁹ and that the fat of the food may have some influence on the phospholipids of the tissues.¹⁵² McCollum¹¹⁹ found with hens that on a fat-free diet the phospholipids of egg yolk had iodine values of 34 to 35 while on normal mixed diet the values were 63 to 64.

The advantages of the phospholipids as intermediate stages in the utilization of the fats are obvious. They mix readily with blood plasma or even with distilled water, forming a dispersion which, while not a true solution, provides a convenient means of transport of the insoluble fats in a watery medium such as the blood and tissues of the animal body. They readily form loose combinations with various substances such as salts, glucose, and proteins; indicating that they may be a very important stabilizing factor in the complex of living protoplasm. They are chemically more reactive than the fats, undergoing hydrolysis (to at least a considerable extent) more readily and are also more readily oxidizable, which may mean no more than that the fatty acids they contain are more readily oxidized than those of the fats.

The evidence available is not sufficient to indicate more than a good possibility that the phospholipids are intermediate stages in the utilization of the fats; and Sinclair's work on the rate of change of tissue phospholipids goes to show that in most tissues, at least, the phospholipids have only a remote connection with the metabolizing fat.

Regarding other early stages in the working up of the long chains of the fatty acids, the theory of Leathes is practically universally accepted. The fat is transported to the liver, unsaturated unions are there introduced into the fatty acids and possibly there, too, the complex compounds of fatty acids with phosphorus and nitrogen (phospholipids) are built up.¹⁰⁰ The data on which Leathes based his theory are as follows:

Hartley⁹⁹ found that the fatty acids of the liver, kidney and heart of several animals (including man) were to a considerable extent acids of a series more unsaturated than oleic acid, having iodine absorption values of 115 to 135; while the fatty acids of the fat stores in the different animals ranged between 35 and 65. In the latter part of his work, he paid particular attention to the identification of the fatty acids in these organs and found that in addition to the saturated acids, palmitic and stearic, which composed about half the acids, there were present an oleic acid with the double bond between the sixth and

seventh carbon atoms, a linolic acid ($C_{18}H_{32}O_2$) with the double bonds between the sixth and seventh and ninth and tenth carbons, and an acid with four double bonds, $C_{20}H_{32}O_2$, which has since been called by Levene *arachidonic acid*. Arachidonic acid was present to the extent of about 10 per cent of the total fatty acids while there was more linolic than oleic acid. Hartley also found that the unsaturated acids were mainly combined in lecithin and similar complex substances.

Other facts which engaged Leathes' attention were that in poisoning with phosphorus, chloroform, phloridzin, mineral acids, etc., in starvation and in many pathologic conditions (in which the important factor may have been starvation), the total fatty acid content of the liver which was not ordinarily more than 3 per cent of the moist weight might reach values up to 20 per cent.^{71, 83} It was also shown that the greater the fatty acid content of the liver the lower was the iodine value and the more nearly it approached the values for stored fat, indicating that the extra fat in liver, under these conditions, was fat brought from the depôts.

Putting together these observations of mobilization of fat to the liver and the presence there of an unusual proportion of unsaturated acids, Leathes made the generalization that transport of fat to the liver was for the purpose of desaturation and was a normal step in fat utilization. Direct evidence of the desaturating power of the liver was obtained by first determining the iodine value of the fatty acids of the liver of rats on a normal diet, then feeding a fat of relatively high iodine value (cod liver oil) and again examining the liver fatty acids.⁹⁹ They were found to have an iodine value considerably higher than that of normal liver fatty acids and higher (over 30 per cent) than that of the cod liver oil fed. These experiments showed also that during fat feeding there was an increase of fat in the liver. Similar results were obtained by others.^{87, 153} Hartley had stated that the unsaturated acids of liver were mainly in combination with lecithin and similar complex substances. Kennaway and Leathes,⁹⁰ and recently Bloor and Snider,²⁸ were able to show that the acetone soluble fraction of the lipids of liver—consisting mainly of fat—contained fatty acids with iodine values much higher than those of adipose tissue (often double the values or more); indicating that the desaturating power of the liver was not limited to the phospholipids but extended to the fats also.

Leathes did not try to exclude the possibility of desaturation or phosphorization in other organs and tissues, but believed that both processes and especially desaturation were carried out mainly in the liver. The evidence in support of his theory regarding these early stages of fatty acid metabolism is perhaps not as extensive as might be desired; but as far as it goes gives good support to his modestly expressed generalization.

Very little is known about the extent to which desaturation is car-

ried. In the blood and most animal tissues, the most unsaturated acid found in quantity is a tetra-unsaturated acid identified by Hartley and by Levene¹¹⁰ as arachidonic acid. Not more than 10 per cent of the acids were found to consist of this one. An acid with three double bonds apparently does not exist in measurable amounts, except in the brain,⁶¹ while two bond acids and of course single bond acids are prevalent. Acids more unsaturated than the four bond acid have been reported only in the brain.

The ability of the animal organism to desaturate the fatty acids has been brought into question because of the discovery of a deficiency disease by Burr and Burr,³² brought on in animals by the lack of linolic and linolenic acids in the food. It is obvious that if the animal body could desaturate fatty acids readily there could be no lack of these essential acids. However, Sinclair¹⁵³ has pointed out that in rats dying of this disease there is still arachidonic acid present in the phospholipids. It may be that the ability to desaturate is specific for certain acids.

Regarding the final stages in the breakdown of the fatty acid chains the prevailing conception is that they are first oxidized at the β -carbon atom from the carboxyl group, producing β -hydroxy- or more probably β -ketonic acids, and then lose the terminal pair of carbon atoms yielding acetic acid and a fatty acid of two less carbons, the process being repeated until the chain is destroyed. The evidence is based largely on results obtained with phenyl derivatives of the fatty acids, by the use of which it has been shown that the fatty acid side-chains are oxidized away in this manner.⁹³ The conclusion that this is the normal method of oxidation of the fatty acid chains is supported by much other evidence. Thus Embden^{45, 46} and his coworkers perfused surviving livers with blood containing even-numbered carbon fatty acids, containing 6, 8, 10 carbon atoms obtaining diacetic acid—which could have been produced only by β -oxidation. Odd-numbered acids did not yield diacetic acid.

In various conditions, which may be grouped under the general head of lack of available carbohydrate (starvation, diabetes, continued vomiting, etc.) the acetone compounds, acetone, diacetic and β -oxybutyric acids are excreted in the urine. These are fatty acid derivatives oxidized in the β -position and are regarded as originating mainly in the fatty acids of the fats. The objection that α -oxidation and not β -oxidation is the common result of oxidation of the fatty acids *in vitro* was answered by the experiments of Dakin³⁷ who showed that when neutralized butyric acid was oxidized *in vitro* under approximately the conditions in the living body; *i. e.*, at about body temperature and with hydrogen peroxide, diacetic acid and acetone were obtained. When the reaction was carried out at higher temperatures the diacetic acid was converted into acetone by loss of carbon dioxide. This reaction was extended by him to higher fatty acids and it was found that every normal fatty acid when neutralized and warmed

with hydrogen peroxide gave the corresponding ketone containing one less carbon atom. This reaction demonstrated the production of β -oxidation *in vitro* in the clearest fashion. No good objection has yet been raised to the theory, and it remains as the best explanation of the final stages of the oxidation of the fatty acids. Other notable efforts in this direction are those of Clutterbuck and Raper³⁵ who were able to show that there was some α - and γ -oxidation along with the β -type; and most recently Witzemann,¹⁷⁷ whose work goes to show that the oxidation may be at either the α - or β -carbon depending on conditions, mainly on the reaction of the mixture.

Accepting desaturation as the first stage in the catabolism of the fatty acids and β -oxidation as the final stage, what can be said about the steps coming between? The answer is—practically nothing.

The mechanism of oxidation of the fatty acids as far as is known at the present time may be summed up as follows: The stable fatty acids of the food or stores are made less stable by desaturation, a process which takes place mainly if not entirely in the liver. Further reactivity may be conferred by phosphorylation of the glycerides. The long chains are then shortened two carbon atoms at a time by β -oxidation, probably with the aid of organic peroxides; at least to the four carbon stage, where a different type of oxidation involving the simultaneous oxidation of glucose appears to be required in most animals. The two carbon fragments are oxidized to carbon dioxide and water.

Ketogenesis and Antiketogenesis.—In the absence of available carbohydrate, many animals (including man) are unable to oxidize completely the fatty acids past the four carbon stage, and the unburned products, diacetic acid, β -hydroxybutyric acid and acetone (the *acetone* or *ketone bodies*) appear in the excretions. Two of these substances are fairly strong acids and all three are believed by some to be toxic in addition to their acidity.³ The formation in quantity of these acids which must be neutralized before excretion, puts a severe strain on the ability of the organism to supply alkali for their neutralization, and results are often fatal owing to depletion of the fixed alkalis necessary for respiration. The dog, and probably carnivorous animals in general, are immune to this type of poisoning; a result undoubtedly of long adaptation to the lack of carbohydrate and the presence of large amounts of fat in their diet. Instances of similar adaptation of human beings and other animals to a high fat diet are available in the literature. Thus Wigglesworth,¹⁷¹ in experiments on rats with a straight fat diet, found marked ketosis which reached a maximum on the third day, then subsided and adaptation was complete on the fifth day. Sodium bicarbonate (6 per cent) prevented the adaptation, causing an increased output of lactic and β -hydroxybutyric acid, indicating that much alkali may interfere with the normal oxidation of fat.

Folin⁵² found that obese individuals when fasted excreted fewer acetone compounds on the second and succeeding fasts than on

the first one. The amount of actual or potential carbohydrate necessary in the diet of human beings in order to avoid this "acidosis" or "ketogenesis" has been the subject of many investigations, of which the most extensive have been those of Woodyatt¹⁷⁸ and Shaffer.¹⁵⁰

Without going into the elaborate details of this work, which has been reviewed by Shaffer,¹⁵⁰ the results may be summed up as follows: One molecule of glucose or its equivalent in other substances which yield sugar in metabolism (*antiketogenic substances*) is theoretically able to secure the complete combustion of two molecules of fatty acid or other substances yielding ketone compounds (*ketogenic substance*); but owing, probably, to uneven distribution and uneven metabolism in different parts of the body it is necessary, in order to be certain of avoiding the production of the acetone derivatives, to allow one molecule of antiketogenic substance, such as glucose, in the diet to one molecule of ketogenic substance such as fatty acid. These results have already been applied satisfactorily in clinical practice,^{68, 80, 172} which is proof of their essential soundness. Shaffer has also contributed much toward the elucidation of the manner in which the carbohydrates assist in the combustion of these fragments of the fatty acids, finding that the combustion of diacetic acid in the presence of glucose is probably preceded by a condensation of the Knövenagel type between some derivative of glucose and the diacetic acid, the condensation product being much more easily oxidized than the diacetic acid alone.^{149, 151}

MacCallum¹¹⁸ has offered an explanation of the facts of acid production from fats which merits attention because of its simplicity and because of the ease with which it may be adapted to explain the various phenomena of acid production. It is that the ability of the human being to burn fat is limited and when the limit is exceeded, combustion is incomplete and the acetone bodies result. All that is necessary to prevent acid production from fat is to supply enough other fuel so that the fat-burning limit is not exceeded. Animals, including the human being, may be trained or adapted to the combustion of larger amounts of fat. The dog does not ordinarily form acids from fat because of racial adaptation to its use.

Acid Intoxication.—The condition of acid intoxication is in man almost always associated with incomplete combustion of fatty acid residues, due to depletion of fixed alkali noted above; and poisoning with these organic acids is not an infrequent occurrence, although confined largely to one disease—diabetes mellitus. They are not formed in appreciable amounts in the organism as long as a minimum quantity of carbohydrate is being utilized. This carbohydrate may be available as such or be formed from protein. In the normal individual, a lack of available carbohydrate occurs practically only in fasting; but in the diabetic it occurs as the result of inability to utilize carbohydrate even though adequate amounts may be supplied.

Excretion of these acids in man often reaches 15 to 20 Gm. per

day; and as much as 150 to 200 Gm. has been claimed. β -Hydroxybutyric is normally 60 to 80 per cent of the total.⁸⁹ In normal blood Marriott¹²³ found about 4 mg. of β -hydroxybutyric and 1.5 mg. of acetone and diacetic together; while in blood of diabetic coma the figures were 45 and 28 mg. per 100 cc. of blood, respectively.

The tissues, and especially the blood, are kept at a reaction very near the neutral point (pH 7.3 to 7.4); and the extreme variations consistent with life are only a few tenths either way. In addition to preserving the reaction within these narrow limits, provision must be made for a certain reserve of fixed alkali necessary to take care of the carbon dioxide formation and excretion. To keep the reaction of blood and tissues within these narrow limits of reaction noted, and to keep a sufficient "alkali reserve" in the face of acid production, various devices are made use of in the animal economy. The more important ones are (1) the excretion of a urine as acid as possible—the limit in humans appears to be a pH of about 5;⁷⁴ (2) the neutralization of the acids by ammonia, making use for the purpose of nitrogen which would otherwise be excreted as urea;⁵⁰ (3) the use of excess alkali in the food, and, as a last resort, the use of the fixed alkali of the blood and tissues down to a point where respiration can no longer be carried on. Animals vary a great deal in their ability to protect their fixed alkali. Herbivorous animals have very little power of resistance, while carnivorous animals (such as the dog) are very resistant. Man comes in between these two extremes; and, when carbohydrate is not present in the food or cannot be made available from it (as in diabetes) he is in danger of death, due to depletion of the alkali necessary for respiration.

Intermediary Metabolism of Other Lipids.—Very little is known regarding the behavior of the other lipid substances in metabolism.

Cholesterol appears to be necessary for life and is either supplied in the food or synthesized as needed. Other sterols, apparently, cannot be used. There is evidence that when the intake is inadequate, cholesterol is retained and used over again.⁴³

Fat Excretion.—Fatty material is normally present in the feces of animals, and it is ordinarily regarded as material that has escaped digestion. Evidence has, however, accumulated which goes to show that it is not unabsorbed material but represents largely an excretion, whether directly from the blood by way of the intestinal secretions, or indirectly as desquamated cells from the intestinal tract.^{75, 133, 134} The possibility of its origin in the bacteria present in the intestine and composing a notable percentage of the fecal mass cannot be excluded; but since the bacteria usually present in the intestine contain very little fat, they are probably not of great importance as sources of fecal fat. Recent work has shown that the lipid output in the feces, while to some extent related to the amount and kind of fat in the food, is, in general, remarkably independent of it and approaches in composition that of the blood.^{79, 159} Under certain conditions such as lack of

bile or pancreatic secretion, and when the food contains a large amount of bulky indigestible material, fat may escape digestion or absorption. The excretion of fatty material is almost entirely as fatty acids and sterols, very little unhydrolyzed fat appearing except when the secretion of bile is stopped.

Excess of cholesterol in the food is largely excreted in the feces, partly *via* the bile but largely directly into the intestine. Some of it is excreted as such, some in reduced forms such as coprosterol.

FATS IN THE DIET

Fats are the most concentrated of the foodstuffs, about 90 per cent of their weight being available for combustion. Their energy value is consequently the highest of all the foodstuffs.

The heats of combustion of the natural food fats vary from 9 calories per gram for coconut oil to 9.5 calories for goose fat. The average value used in computations of energy in animals is 9.3.

Because of their concentrated nature, they are made use of largely in plants and animals as a form in which energy may be stored. In the human diet they are valuable as convenient concentrated sources of energy, reducing the bulk of the food intake, and adding the factors of flavor and palatability as well as certain valuable vitamins. In view of our present knowledge of tissue constituents, the statement formerly made regarding them that "they cannot serve for the up-building or renewal of tissues" must be revised, since such substances as lecithin are probably as important constituents of tissue as protein.

The fact that fats can be synthesized from other foodstuffs, such as carbohydrates, has raised the question as to whether they can be dispensed with in the diet. Experiments on this point have been carried out by various workers,^{40, 78} and the more recent experiments by McAmis, Anderson and Mendel¹¹⁶ and Burr and his associates³³ give overwhelming evidence that certain fatty acids are necessary in the food, and if they are lacking continued life is impossible. The specific substance necessary appears to be linolic acid.

In starvation, the carbohydrate stores (glycogen) are exhausted in the first two or three days, and after that the energy requirement falls chiefly on the fat together with a minimum of body protein, and the animal lives as long as his fat stores last. When these are exhausted, body protein alone is left; and since its energy value is less than half that of fat, and is, moreover, as tissue mixed with about three times its weight of water, the animal soon dies. The composition of the organs most essential to life, such as the heart, kidney and brain, is preserved almost to the end.

MILK FAT

The composition of the milk fat of animals is quite different from the stored fat in the same animals. It contains representatives of practically all the saturated acids from butyric acid, which is C_4 , to ligno-

ceric acid, which is C_{24} ; and possibly higher. The content of the short-chain fatty acids is notably higher than in any other fat of the animals. Recent work by Bosworth and Brown³⁰ indicates that the fatty acid mixture of cow's milk fat is almost hopelessly complex. They find, in addition to the saturated acids mentioned, a considerable variety of unsaturated acids.

The relation of the milk fat to the fat of the food appears to vary in different animals.^{55, 128} In the case of carnivora and omnivora, food fat may appear promptly in the milk; while in the case of the herbivora the food fat appears to have very little direct influence on the milk fat; and the fat of the milk appears to be formed largely from carbohydrate. According to Smith, Wells and Ewing,¹⁵⁸ the milk fat of cows is influenced by food fat but the effect is not immediate, requiring some days to become evident. The participation of the blood lipids in the formation of milk fat is indicated by the work of Meigs, Blatherwick and Cary,¹²⁷ who find a definite relation between the disappearance of phospholipid from the blood in the milk gland and the formation of milk fat. Other relations of the blood lipids to milk fat have been pointed out by Maynard and his associates.¹²⁶

VITAMINS ASSOCIATED WITH FATS

Three of the vitamins are associated with fats in natural products and so enhance their importance in the diet. These are the fat-soluble or growth vitamin A, the antirachitic vitamin D, and the reproductive or fertility vitamin E. For more specific information on the occurrence and properties of the vitamins the reader is referred to the chapter on these substances (Chapter IX).

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REFERENCES

1. Abderhalden, E.: *Biochemisches Handlexikon* (1911), 3, p. 178.
2. Abderhalden, E., and Eichwald, E.: *Ber.*, 48, 1847 (1915).
3. Allen, F. M., and Wishart, M. B.: *J. Metabolic Research*, 4, 613 (1923).
4. Amberger, C., and Bromig, K.: *Biochem. Z.*, 130, 252 (1922).
5. Anderson, R. J.: *J. Biol. Chem.*, 83, 505 (1929).
6. Anderson, R. J.: *J. Biol. Chem.*, 55, 611 (1923).
7. Anderson, W. E.: *J. Biol. Chem.*, 63, xlvii (1925).
8. Anderson, W. E., and Mendel, L. B.: *Proc. Soc. Exp. Biol. Med.*, 21, 436 (1924).
9. Armstrong, H. E., and Gosney, H. W.: *Proc. Roy. Soc. (London)*, 88B, 176 (1915).
10. Atkinson, H. V., Rapport, D., and Lusk, G.: *J. Biol. Chem.*, 53, 155 (1922).
11. Bailey, C. H.: *J. Exp. Med.*, 23, 69 (1916).
12. Bang, I.: *Biochem. Z.*, 91, 104 (1918).
13. Banks, A., and Hilditch, T. P.: *Biochem. J.*, 26, 298 (1932).
14. Berend, N.: *Biochem. Z.*, 260, 490 (1933).
15. Bergmann, M., and Sabetay, S.: *Z. physiol. Chem.*, 137, 47 (1924).
16. Best, C. H., and Huntsman, M. E.: *J. Physiol.*, 75, 405 (1932).
17. Blatherwick, N. R., Medlar, E. M., Bradshaw, P. J., Post, A. L., and Sawyer, S. D.: *J. Biol. Chem.*, 103, 93 (1933).
18. Blix, G.: *Studies on Diabetic Lipemia* (Lund, 1925); also *Acta Med. Scand.*, 64, 142 (1926).
19. Bloor, W. R.: *J. Biol. Chem.*, 11, 429 (1912).
20. Bloor, W. R.: *J. Biol. Chem.*, 16, 517 (1914).

21. Bloor, W. R.: *J. Biol. Chem.*, **24**, 447 (1916).
22. Bloor, W. R.: *J. Biol. Chem.*, **29**, 437 (1917).
23. Bloor, W. R.: *J. Biol. Chem.*, **49**, 201 (1921).
24. Bloor, W. R.: *Physiol. Rev.*, **2**, 92 (1922).
25. Bloor, W. R.: *J. Biol. Chem.*, **56**, 711 (1923).
26. Bloor, W. R.: *J. Biol. Chem.*, **59**, 543 (1924).
27. Bloor, W. R.: *J. Biol. Chem.*, **63**, 1 (1925).
28. Bloor, W. R., and Snider, R. H.: *J. Biol. Chem.*, **87**, 399 (1930).
29. Bondi, S., and Neumann, A.: *Wiener klin. Wochschr.*, **23**, 734 (1910).
30. Bosworth, A. W., and Brown, J. B.: *J. Biol. Chem.*, **103**, 115 (1933).
31. Bradley, H. C.: *J. Biol. Chem.*, **13**, 407 (1913).
32. Burr, G. O., and Burr, M. M.: *J. Biol. Chem.*, **82**, 345 (1929).
33. Burr, G. O., Burr, M. M., and Miller, E. S.: *J. Biol. Chem.*, **97**, 1 (1932).
34. Chanutin, A., and Ludewig, S.: *J. Biol. Chem.*, **102**, 57 (1933).
35. Clutterbuck, P. W., and Raper, H. S.: *Biochem. J.*, **19**, 385 (1925).
36. Cremer, M.: *Münch. med. Wochschr.*, **49**, 944 (1902).
37. Dakin, H. D.: *J. Biol. Chem.*, **4**, 77, 91 (1908).
38. Degkwitz, R.: *Ergebnisse Physiol.*, **32**, 821 (1931).
39. Doree, C.: *Biochem. J.*, **4**, 72 (1909).
40. Drummond, J. C., and Coward, K. H.: *Lancet*, **201**, 698 (1921, pt. 2).
41. Eckstein, H. C.: *J. Biol. Chem.*, **62**, 737 (1925).
42. Eichholtz, F.: *Biochem. Z.*, **144**, 66 (1924).
43. Ellis, G. W., and Gardner, J. A.: *Proc. Roy. Soc. (London)*, **84B**, 461 (1912).
44. Ellis, N. R., and Isbell, H. S.: *J. Biol. Chem.*, **69**, 219 (1926).
45. Embden, G., and Marx, A.: *Beitr. chem. Phys. Path.*, **11**, 318 (1908).
46. Embden, G., Salomon, H., and Schmidt, F.: *Beitr. chem. Phys. Path.*, **8**, 129 (1906).
47. Erdmann, E., and Bedford, F.: *Ber.*, **42**, 1324 (1909).
48. d'Errico, G.: *Arch. fisiol.*, **4**, 513 (1906-7).
49. Falk, K. G.: *The Chemistry of Enzyme Action* (1924).
50. Fiske, C. H., and Sokhey, S. S.: *J. Biol. Chem.*, **63**, 309 (1925).
51. Foà, C.: *Arch. ital. biol.*, **63**, 229 (1915).
52. Folin, O., and Denis, W.: *J. Biol. Chem.*, **21**, 183 (1915).
53. Frank, O.: *Z. Biol.*, **36**, 568 (1898).
54. Fraser, M. T., and Gardner, J. A.: *Proc. Roy. Soc. (London)*, **82B**, 559 (1910).
55. Gage, S. H., and Fish, P. A.: *Am. J. Anat.*, **34**, 1 (1924).
56. Gardner, J. A., and Fox, F. W.: *Biochem. J.*, **18**, 1058 (1924).
57. Gardner, J. A., and Williams, M.: *Biochem. J.*, **15**, 363 (1921).
58. Geelmuyden, C.: *Ergebnisse Physiol.*, **22**, 51 (1923).
59. Greenbank, G. R., and Holm, G. E.: *Ind. Eng. Chem.*, **16**, 598 (1924).
60. Gray, H.: *Am. J. Med. Sci.*, **168**, 35 (1924).
61. Grey, E. C.: *Biochem. J.*, **7**, 148 (1913).
62. Grigaut, A., and L'Huilliere, A.: *Compt. rend. soc. biol.*, **73**, 202 (1912).
63. Grün, A., and Kade, F.: *Ber.*, **45**, 3367 (1912).
64. Grün, A., and Limpacher, R.: *Chem. Umschau*, **30**, 246 (1923); *Chem. Abstracts*, **18**, 536 (1924).
65. Grün, A., and Schacht, P.: *Ber.*, **40**, 1778 (1907).
66. Hamsik, A.: *Z. physiol. Chem.*, **71**, 238 (1911).
67. Hanes, F. M.: *J. Exp. Med.*, **16**, 512 (1912).
68. Hannon, R. R., and McCann, W. S.: *Bull. Johns Hopkins Hosp.*, **33**, 128 (1922).
69. Hartley, P.: *J. Physiol.*, **36**, 17 (1907).
70. Hartley, P.: *J. Physiol.*, **38**, 353 (1909).
71. Hartley, P., and Mavrogordato, A.: *J. Path. Bact.*, **12**, 371 (1908).
72. Hausmann, W.: *Beitr. chem. Physiol. Path.*, **6**, 567 (1905).
73. Same as 72.
74. Henderson, L. J., and Palmer, W. W.: *J. Biol. Chem.*, **13**, 393 (1912-13).
75. Hermann, L.: *Arch. ges. Physiol.*, **46**, 93 (1890).
76. Hesse, O.: *Ann.*, **192**, 175 (1878).
77. Hilditch, T. P.: *J. Soc. Chem. Ind.*, **52**, 169 (1933).
78. Hindhede, M.: *Molkerei-Ztg.*, **28**, 152; *Chem. Abstracts*, **14**, 423 (1920).
79. Holmes, A. D., and Kerr, R. H.: *J. Biol. Chem.*, **58**, 377 (1923).
80. Hubbard, R. S., and Nicholson, S. T.: *J. Biol. Chem.*, **53**, 209 (1922).
81. Hueck, W., and Wacker, L.: *Biochem. Z.*, **100**, 84 (1919).
82. Hürthle, K.: *Z. physiol. Chem.*, **21**, 331 (1895-96).

83. Imrie, C. G.: *J. Path. Bact.*, **19**, 245 (1914).
84. Imrie, C. G., and Graham, S. G.: *J. Biol. Chem.*, **41**, xlviii (1920).
85. Jackson, F. M.: *J. Ministry Agr.*, **39**, 261 (1923); *Chem. Abstracts*, **17**, 2920 (1923).
86. Janney, N. W.: *J. Biol. Chem.*, **20**, 321 (1915).
87. Joannovics, G., and Pick, E. P.: *Wiener klin. Wochschr.*, **23**, 573 (1910).
88. Jordan, W. H., Hart, E. B., and Patten, A. J.: *Am. J. Physiol.*, **16**, 268 (1906).
89. Kennaway, E. L.: *Biochem. J.*, **8**, 355 (1914).
90. Kennaway, E. L., and Leathes, J. B.: *Proc. Roy. Soc. Med.*, **2**, Path. Sec., 136 (1909).
91. Kerr, R. H., and Sorber, D. G.: *Ind. Eng. Chem.*, **15**, 383 (1923).
92. Klenk, E.: *Z. physiol. Chem.*, **192**, 217 (1930); **200**, 51 (1931).
93. Knoop, F.: *Beitr. chem. Physiol. Path.*, **6**, 150 (1905).
94. Knudson, A.: *J. Biol. Chem.*, **32**, 337 (1917).
95. Knudson, A.: *J. Biol. Chem.*, **45**, 255 (1921).
96. Kreis, H., and Hafner, A.: *Ber.*, **36**, 2766 (1903).
97. Kumagawa, M., and Suto, K.: *Biochem. Z.*, **8**, 212 (1908).
98. Langworthy, C. F.: *Ind. Eng. Chem.*, **15**, 276 (1923).
99. Leathes, J. B., and Meyer-Wedell, L.: *J. Physiol.*, **38**, xxxviii (1909).
100. Leathes, J. B., and Raper, H. S.: *The Fats* (1925).
101. Lee, F. C.: *Johns Hopkins Hosp. Bull.*, **33**, 21 (1922).
102. Lemeland, P.: *Compt. rend. soc. biol.*, **85**, 839 (1921).
103. Lemeland, P.: *Bull. soc. chim. biol.*, **5**, 110 (1923).
104. Levene, P. A.: *J. Biol. Chem.*, **24**, 69 (1916).
105. Levene, P. A., and co-workers: Various articles in the *J. Biol. Chem.* from 1917 to date.
106. Levene, P. A., and Rolf, I. P.: *J. Biol. Chem.*, **51**, 507 (1922).
107. Levene, P. A., and Rolf, I. P.: *J. Biol. Chem.*, **54**, 91, 99 (1922).
108. Levene, P. A., and Rolf, I. P.: *J. Biol. Chem.*, **60**, 677 (1924).
109. Levene, P. A., and Rolf, I. P.: *J. Biol. Chem.*, **62**, 759 (1924).
110. Levene, P. A., and Simms, H. S.: *J. Biol. Chem.*, **51**, 285 (1922).
111. Levene, P. A., Rolf, I. P., and Simms, H. S.: *J. Biol. Chem.*, **58**, 859 (1924).
112. Lewkowitsch, J.: *Ber.*, **25**, 65 (1892).
113. Lewkowitsch, J. I.: *Chemical Technology and Analysis of Oils, Fats, and Waxes*, **1**, 117 (1921).
114. Loevenhart, A. S.: *Am. J. Physiol.*, **6**, 331 (1902).
115. Lusk, G.: *The Elements of the Science of Nutrition* (1921).
116. McAmis, A. J., Anderson, W. E., and Mendel, L. B.: *J. Biol. Chem.*, **82**, 247 (1929).
117. MacArthur, C. G., and Burton, L. V.: *J. Am. Chem. Soc.*, **38**, 1375 (1916).
118. MacCallum, A. B.: *Can. Med. Assoc. J.*, **22**, 3 (1930).
119. McCollum, E. V., Halpin, J. G., and Drescher, A. H.: *J. Biol. Chem.*, **13**, 219 (1912).
120. McClure, C. W., and Huntsinger, M. E.: *J. Biol. Chem.*, **76**, 1 (1928).
121. MacLean, H., and MacLean, I. S.: *Lecithin and Allied Substances, the Lipins* (1927).
122. McMeans, J. W.: *J. Med. Research*, **33**, 481 (1916).
123. Marriott, W. M.: *J. Biol. Chem.*, **18**, 507 (1914).
124. Mayer, A., and Schaeffer, G.: *Compt. rend.*, **157**, 156 (1913).
125. Mayer, A., and Schaeffer, G.: *J. physiol. path. gén.*, **16**, 203 (1914).
126. Maynard, L. A., Harrison, E. S., and McCay, C. M.: *J. Biol. Chem.*, **92**, 263 (1931).
127. Meigs, E. B., Blatherwick, N. R., and Cary, C. A.: *J. Biol. Chem.*, **37**, 1 (1919).
128. Mendel, L. B., and Daniels, A. L.: *J. Biol. Chem.*, **13**, 71 (1912).
129. Mills, L. H.: *Arch. Internal Med.*, **7**, 694 (1911).
130. Minkowski, O.: *Klin. Wochschr.*, **27**, 333 (1890).
131. Moore, B., and Parker, W. H.: *Proc. Roy. Soc. (London)*, **68B**, 64 (1901).
132. Moore, B., and Rockwood, D. P.: *J. Physiol.*, **21**, 58 (1897).
133. Müller, F.: *Arch. path. Anat. Virchow's*, **131** Suppl., 106 (1893).
134. Müller, F.: *Z. Biol.*, **20**, 327 (1884).
135. Mueller, J. H.: *J. Biol. Chem.*, **22**, 1 (1915); **27**, 463 (1916).
136. Munk, I., and Rosenstein, A.: *Arch. path. Anat. Virchow's*, **123**, 230, 484 (1891).

137. Palmer, L. S.: *Carotinoids and Related Pigments; the Chromolipoids* (1922).
138. Palmer, L. S., and Eckles, C. H.: *J. Biol. Chem.*, 17, 191, 211, 223, 237.
245 (1914).
139. Parnas, J.: *Biochem. Z.*, 56, 17 (1913).
140. Pettenkofer, M. v., and Voit, C.: *Z. Biol.*, 9, 1 (1873).
141. Rabbeno, A.: *Arch. sci. med.*, 38, 259 (1914).
142. Raper, H. S.: *J. Biol. Chem.*, 14, 117 (1913).
143. Reed, L. L., Anderson, W. E., and Mendel, L. B.: *J. Biol. Chem.*, 96, 313 (1932).
144. Rosenmund, K. W., and Kuhnenn, W.: *Z. Untersuch. Nahr.-Genussm.*, 46, 154 (1923).
145. Rowland, R. S.: *Oxford Medicine*, vol. 4, 214 (3).
146. Rubow, V.: *Arch. exp. Path. Pharmacol.*, 52, 173 (1905).
147. Sabatier, P.: *Catalysis in Organic Chemistry* (1923), p. 937.
148. Schönheimer, R., and Dam, H.: *Z. physiol. Chem.*, 215, 59 (1933).
149. Shaffer, P. A.: *J. Biol. Chem.*, 47, 433 (1921).
150. Shaffer, P. A.: *Harvey Lectures*, 18, 105 (1923).
151. Shaffer, P. A., and Friedemann, T. E.: *J. Biol. Chem.*, 61, 585 (1924).
152. Shioji, E.: *J. Biochem. (Japan)*, 4, 43 (1924).
153. Sinclair, R. G.: *J. Biol. Chem.*, 82, 117 (1929).
154. Sinclair, R. G.: *J. Biol. Chem.*, 92, 245 (1931).
155. Sinclair, R. G.: *J. Biol. Chem.*, 97, xxxiv (1932).
156. Slowtsoff, B.: *Beitr. chem. Physiol. Path.*, 7, 508 (1906).
157. Smith, C. S.: *J. Cut. Diseases*, 36, 436 (1918).
158. Smith, F. H., Wells, C. A., and Ewing, P. V.: *Georgia Exp. Station, Bull.* 122, June, 1916.
159. Sperry, W. M., and Bloor, W. R.: *J. Biol. Chem.*, 60, 261 (1924).
160. Steuart, D. W.: *Analyst*, 48, 155 (1923).
161. Tangl, H., and Berend, L.: *Biochem. Z.*, 220, 234 (1930).
162. Taylor, A. E.: *J. Med. Research*, 9, 59 (1903).
163. Terroine, E. F.: *J. physiol. path. gén.*, 16, 386 (1914).
164. Terroine, E. F.: *J. physiol. path. gén.*, 16, 408 (1914).
165. Thaysen, Th. E. H.: *Biochem. Z.*, 62, 89 (1914).
166. Thierfelder, H., and Klénk, E.: *Die Chemie der Cerebroside und Phosphatide* (1930).
167. Twitchell, E.: *Ind. Eng. Chem.*, 6, 564 (1914); 9, 581 (1917).
168. Verzár, F.: *Nutrition Abstracts Rev.*, 2, 441 (1933).
169. Verzár, F., and Kuthy, A.: *Biochem. Z.*, 205, 369 (1929); 210, 265 (1929).
170. Welter, A.: *Z. angew. Chem.*, 24, 385 (1911).
171. Wigglesworth, V. B.: *Biochem. J.*, 18, 1203 (1924).
172. Wilder, R. M.: *J. Am. Med. Assoc.*, 78, 1878 (1922).
173. Willstätter, R., Haurowitz, F., and Memmen, F.: *Z. physiol. Chem.*, 140 203 (1924).
174. Windaus, A.: *Ber.*, 42, 238 (1909).
175. Windaus, A.: *Z. physiol. Chem.*, 65, 110 (1910).
176. Windaus, A.: *Z. physiol. Chem.*, 213, 147 (1932).
177. Witzemann, E. J.: *J. Biol. Chem.*, 95, 219, 247 (1932).
178. Woodyatt, R. T.: *Arch. Internal Med.*, 28, 125 (1921).
179. Ziegler, K.: *Z. ges. exp. Med.*, 24, 242 (1921).
180. Zucker, T. F.: *Proc. Soc. Exp. Biol. Med.*, 17, 89 (1920).

CHAPTER XXII

THE METABOLISM OF PROTEINS AND AMINO ACIDS

As described in Chapter IV proteins may be thought of as condensation products of α -amino acids, of which at least 21 have been satisfactorily identified among the products of protein hydrolysis.¹ It is not within the scope of the present chapter to discuss the means by which these products are liberated during protein digestion *in vivo*; but consideration will be given to their absorption and their ultimate fate within the organism.

It will not be possible to consider in detail the 21 separate pathways followed by the amino acids in metabolism, partly because these details, in many instances, remain unknown. In others there seems to be a fair degree of conformity to a general pattern which renders unnecessary any detailed treatment of individual cases. There remains, however, a small group of amino acids which are metabolized along pathways of greater complexity or which present features of unusual interest. These will be considered individually. In most of the space at our disposal we shall be obliged to consider the more general aspects of the metabolism of proteins and amino acids.

By way of introduction, it may not be amiss to indicate something of the importance of these compounds in the economy of living things. Perhaps without exception, they constitute the bulk of the organic matter in the living, metabolizing tissues. On this account many writers have been prone to regard vital activity as something which is inseparably associated with the cell proteins. The term "vital activity," as here used, denotes those fundamental properties of all living things which distinguish them from inanimate objects.

It follows, therefore, that the establishment of appropriate nutritive conditions for synthesis of the cell proteins is an important factor in tissue elaboration and growth. As has been known for more than one hundred years,* nitrogen is the most characteristic element in proteins; and so the satisfaction of the nutritive requirement so far as the cell proteins are concerned is largely a matter of providing nitrogen in suitable form. The higher animals, as it happens, are able to employ amino acid nitrogen alone.† In consequence the quality and quantity of the food proteins becomes a matter of prime importance.

* The first quantitative determinations were published by Gay-Lussac and Thenard in 1810.²

† The apparent utilization of ammonium salts and urea in ruminants is thought to depend upon the presence of bacterial and protozoal masses in the rumen. These microorganisms are able to use ammonium salts or urea as sources of nitrogen for growth. The proteins thus elaborated are digested and used by the host on the death of the organisms.

The Protein Requirement.—A close relationship exists between the nitrogen intake and the nitrogen output. The normal adult, under proper dietary conditions, is in a state of nitrogen balance. If his protein intake be suddenly increased, his nitrogen output rises until after several days a new "equilibrium" at a higher level is attained. Conversely, reduction of the protein intake is followed by a lessening of the nitrogen output until a new balance is struck at a lower level.

It is to be understood, however, that there are limits beyond which the nitrogen intake may not vary. The upper limit is dependent, in part, upon the total caloric requirement but is even more subject to individual dietary habits. The normal adult can seldom eat more than 300 Gm. of protein daily without nausea. Eskimos may ingest without discomfort still larger quantities.* When the total caloric requirement is high the protein intake may be substantially increased. Atwater⁴ found that whereas the average adult in the United States ingests 97–113 Gm. of protein daily on a total food intake of about 2500 Calories, the most generously fed (football players) received as much as 220–240 Gm. of protein on a food intake of 6600–7800 Calories. Destitute laborers ate as little as 52 Gm. of protein on a food intake of 1640 Calories.

The lowest level of protein intake at which nitrogen equilibrium can still be maintained is defined as the *physiologic protein minimum*. This may be as low as 0.39 Gm. per kilo per day⁵ though most observers report a somewhat higher value.⁶ The *hygienic protein minimum*, defined as the minimum protein intake compatible with the maintenance of health, is definitely greater. From the experience of Susskind⁷ it must be above 0.5 Gm. per kilo which was his daily intake during twenty-five months. According to E. Voit⁸ it is more than 0.7 Gm. per kilo; but how much greater still remains undetermined.

By the ingestion of diets from which proteins have been almost completely excluded, and with fat and carbohydrate serving as the sole exogenous sources of fuel, the level of protein catabolism may be reduced below the physiologic protein minimum. The lowest level to which it may be brought is defined as the *minimum of protein catabolism*. Values of 0.15 to 0.25 Gm. per kilo per day (calculated from the urinary nitrogen output) have been reported.^{9, 10, 11} It is a measure of the irreducible "wear and tear" of the body proteins—"the unavoidable metabolic waste arising from cell activity." (Fig. 43.)

Resumption of protein ingestion does not lead to a prompt and corresponding increase in nitrogen output. The nitrogen excretion may increase slowly for some days. Balance is eventually restored. The rate of recovery, and the level of output at which the new balance is struck are very dependent upon the quality of protein ingested. Con-

* "If we were to go naked, like certain savage tribes, or if in hunting or fishing we were exposed to the same degree of cold as the Samoyedes, we should be able with ease to consume 10 pounds of flesh, and perhaps a dozen of tallow candles into the bargain, daily. . . ."³

versely, the sudden withdrawal of protein leads to a fall in the level of protein catabolism, the decrease proceeding rapidly for a day or two and then tapering off gradually until the new level is attained (Fig. 44). The rate of decrease depends directly upon the previous level of protein intake.

On the withdrawal of all food, the excretion of nitrogen rapidly decreases; but the level finally attained is not so low as during the ingestion of protein-free diets rich in carbohydrate. The body protein in the fasting subject is spared by the oxidation of fat. Further sparing of protein follows the administration of carbohydrate.

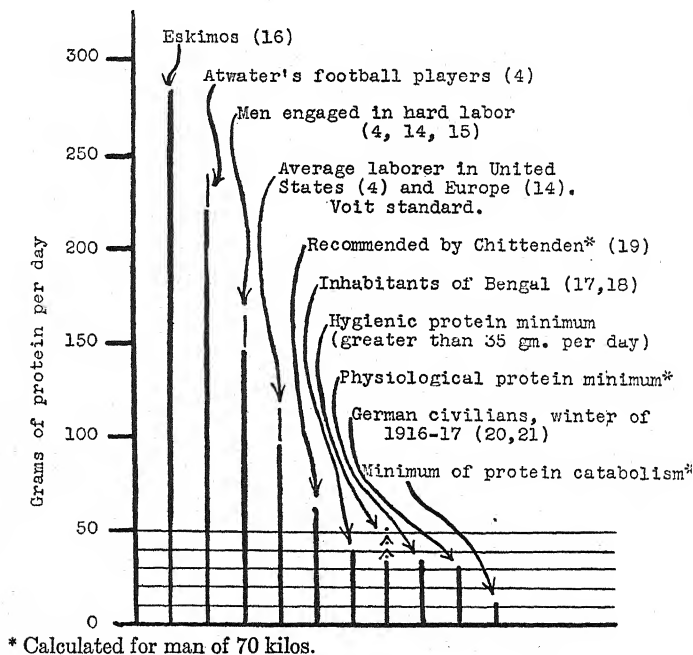


Fig. 43.—Levels of protein metabolism.

Such observations have suggested to many investigators the existence within the organism of two metabolic varieties of protein which differ in their availability for, or ease of, catabolism. The less available of the two is described in the literature as "organ protein,"¹² "organized protein,"¹³ "tissue protein," "living protein," and "stable protein." The terms refer to that fraction which is postulated to be an integral part of the very structure of the body. Under conditions of protein deprivation it is spared to the last.

The other protein fraction, variously described as "circulating," "unorganized," "reserve," "dead," or "labile," is considered to be that fraction which is quickly catabolized within the first few days of protein deprivation, or which, conversely, is seemingly stored by the

organism during the period of "positive nitrogen balance" immediately following return to an adequate level of protein intake (compare Fig. 44).

It is to be understood that these terms have a descriptive significance only. In the light of present knowledge we cannot regard these two metabolic groups of proteins as possessed of characteristic

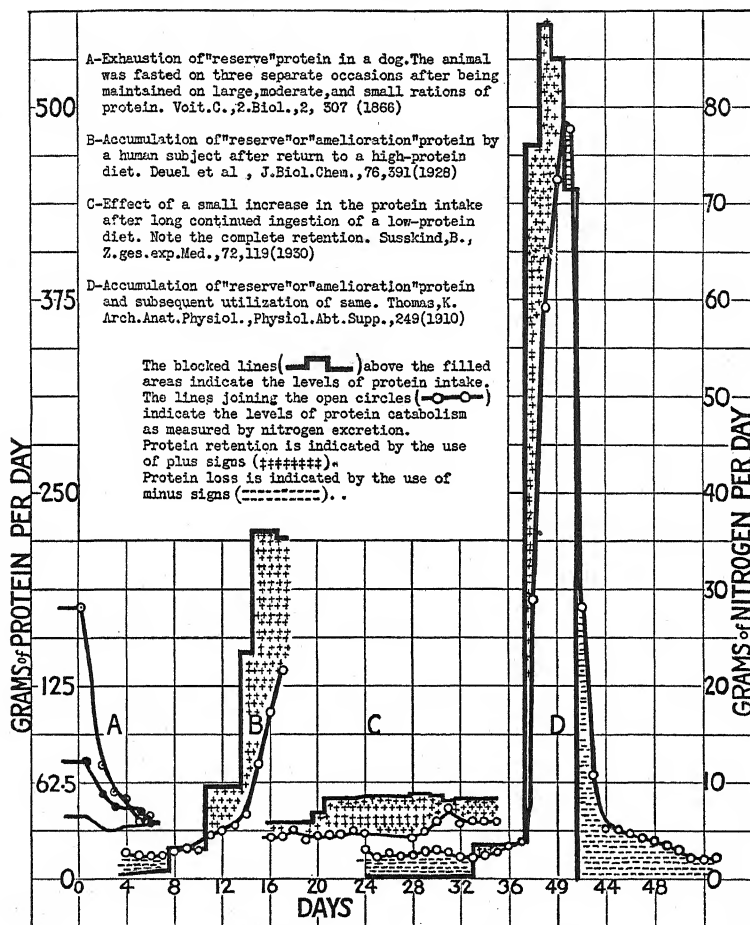


Fig. 44.—"Reserve" protein.

chemical differences. The distinction is of value only in explaining concretely the changes in protein catabolism which take place under different dietary conditions. It assumes a fairly free interconversion of the two types. That there are actual depôts of stored or reserve protein having a metabolic rôle comparable to that of glycogen, is a convenient hypothesis which still lacks adequate experimental verification.

The Principal Nitrogenous Constituents of Urine.—Under usual dietary conditions *urea* accounts for 87 per cent of the nitrogen excreted by the normal adult.²² This abundant excretion was first indicated by Fourcroy and Vauquelin²³ in 1799: "Cette matière animale particulière que nous nommons *urée* . . . forme seule les $\frac{19}{20}$ de ses matériaux." Urea itself had been discovered and first isolated* from urine by Rouelle in 1773.²⁴

Most of the change in nitrogen output as influenced by varying levels of protein intake is due to parallel variations in the rate of urea excretion. This and other observations proves that urea is the principal nitrogenous end-product of protein catabolism.

Another important nitrogenous constituent is *ammonia*. Many of the early demonstrations of its presence do not exclude its secondary formation by the decomposition of urea but an experiment† reported by Boerhaave²⁵ in 1727 is fairly convincing. Berzelius²⁶ recognized ammonia as a primary constituent and Boussingault²⁷ developed a fairly reliable method for its quantitative determination. The normal adult excretes about 0.5–0.8 Gm. per day. Ultimately it must arise from the food or body proteins, although its immediate precursor, whether urea, amino acids, adenylic acid, etc., remains unknown. Its formation by the kidney and the factors governing its excretion are discussed in another chapter. It is sufficient here to mention that the excretion of ammonia is not directly related to the level of protein catabolism.

A nitrogenous constituent which we must consider in greater detail is *creatinine*,³⁰ $\text{HN}=\text{C} \begin{array}{l} \text{NH} \text{---} \text{O} \\ \text{NH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CO} \end{array}$. About 1.4–1.7 Gm. of this substance are formed daily. Its rate of excretion is wholly independent of the level of protein metabolism. This important observation by Folin²⁸ led to the formulation of his now classic distinction between endogenous and exogenous metabolism. Some of the actual experimental findings upon which the theory is based are presented in the accompanying table (p. 617).

The essential observations were that alterations in the protein intake caused changes in the amounts of urea and sulfate excreted which were directly proportional to the changes in the level of protein metabolism. On the contrary, the quantities of creatinine and "neutral sulfur" excreted daily remained unaltered. Parenthetically, it might be mentioned that just as urea is the end-product of protein-nitrogen catabolism, so sulfate, free (inorganic) or combined (etheral), is the

* The substance isolated by Boerhaave, fifty years earlier [(²⁵), Process 87, p. 193] was probably urea.

† "With the rob, or sapa, of recent urine, mix an equal quantity of quicklime in a tall glass vessel, fitted with a receiver, and a violent ebullition, with a great degree of heat will immediately ensue; and at that very instant there will swiftly fly off from the mixture, and pass over into the receiver, a clear liquid or spirit of an exceeding fiery and pungent taste and odor;" Process 86, p. 191.

COMPOSITION OF URINE ON DIETS OF DIFFERENT NITROGEN CONTENT (FOLIN)

| Day. | Volume. | Total N. | Urea N. | NH ₃ N. | Creatinine, N. | Uric acid, N. | Inorganic sulfate. | Ethereal sulfate. | Neutral sulfate. | diet. |
|------|---------|----------|---------|--------------------|----------------|---------------|---------------------|---------------------|---------------------|-----------------------------|
| | cc. | Gm. | Gm. | Gm. | Gm. | Gm. | Gm. SO ₃ | Gm. SO ₃ | Gm. SO ₃ | |
| 1 | 1065 | 14.4 | 12.4 | 0.46 | 0.55 | 0.20 | 2.84 | 0.23 | 0.23 | Milk and eggs. |
| 2 | 980 | 14.8 | 12.9 | 0.45 | 0.49 | 0.18 | 2.71 | 0.20 | 0.16 | |
| 3 | 1000 | 15.7 | 13.6 | 0.54 | 0.48 | 0.14 | 2.82 | 0.22 | 0.23 | |
| 4 | 1080 | 16.8 | 14.7 | 0.54 | 0.43 | 0.16 | 2.99 | 0.19 | 0.16 | |
| 5 | 535 | 9.9 | 8.4 | 0.34 | 0.48 | | 1.11 | 0.15 | 0.19 | Potatoes, starch and cream. |
| 6 | 440 | 7.6 | 5.9 | 0.32 | 0.49 | 0.14 | 0.79 | 0.11 | 0.15 | |
| 7 | 475 | 5.9 | 4.5 | 0.34 | 0.47 | 0.12 | 0.59 | 0.13 | 0.16 | |
| 8 | 405 | 5.4 | 4.0 | 0.33 | 0.51 | 0.13 | 0.68 | 0.07 | | |
| 9 | 380 | 3.5 | 2.0 | 0.33 | 0.48 | 0.13 | 0.41 | 0.09 | 0.15 | |
| 10 | 730 | 4.2 | 2.8 | 0.17 | 0.54 | 0.16 | 0.65 | 0.10 | 0.13 | |
| 11 | 585 | 3.5 | 2.1 | 0.27 | 0.50 | 0.14 | 0.48 | 0.08 | 0.21 | |
| 12 | 1260 | 6.8 | 5.4 | 0.33 | 0.49 | 0.11 | 1.13 | 0.11 | 0.19 | Milk and eggs. |
| 13 | 1375 | 10.9 | 8.9 | 0.36 | 0.53 | 0.14 | 2.76 | 0.20 | 0.24 | |

end-product of protein-sulfur catabolism. "Neutral sulfur," represented by such compounds as cystine, glutathione, and ergothioneine (?)²⁹ cannot be regarded in the same light as urea and sulfate as an oxidation end-product.

To Folin the peculiar constancy in the rates of excretion of "neutral sulfur" and creatinine—their independence of the protein intake—could only mean that they did not arise as products of "exogenous metabolism." It seemed much more plausible to regard creatinine and "neutral sulfur" as products of certain reactions which proceed with constant velocity within the organism without reference or relation to the rate of metabolism of ingested foodstuffs. Just as the latter is described as "exogenous" in character so the former is defined as "endogenous." In summary, therefore, urea and sulfate would be regarded as indicators of the level of *exogenous* protein metabolism and creatinine and neutral sulfur as indicators of *endogenous* protein metabolism. It is difficult, however, to believe that food protein (exogenous) leads to different metabolic products than tissue protein (endogenous). It is better to regard urea and sulfate as end-products of the metabolism of both food and tissue protein, both exogenous and endogenous in origin; and creatinine and neutral sulfur as the products of reactions that are fairly remote from the primary pathways of amino acid catabolism.

Creatinine is derived from *creatine*, $\text{HN}=\text{C} \begin{array}{l} \text{NH}_2 \\ \text{NH}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{COOH} \end{array}$, which is largely localized in muscle, where it occurs partly as a phosphoric acid ester (phosphocreatine). It is not excreted by the

normal male adult; but infants and young children, pregnant women, ruminants, and several other mammals regularly excrete both creatine and creatinine. It is also excreted during starvation, in fevers, wasting diseases, and diseases of carbohydrate metabolism. Rose and Dimmitt³¹ and Benedict and Osterberg³² have shown that the long-continued administration of creatine causes a slowly induced increase in the output of creatinine by the male adult. Proteins and individual amino acids have no such effect, nor do they induce creatinuria in the normal male adult. When creatinuria is already established, however, there is ample evidence that proteins and certain amino acids enhance the output of creatine.³³ It is of interest that the excretion of creatine which is in evidence in certain atrophic diseases of muscle, is definitely increased by the feeding of glycine. In certain cases (myasthenia gravis) a distinct clinical improvement sets in simultaneously. Attractive as it may be, the hypothesis that creatine originates from arginine, $\text{HN}=\text{C} \begin{array}{l} \nearrow \text{NH}_2 \\ \searrow \text{NH} \cdot (\text{CH}_2)_3 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} \end{array}$, is without experimental validity.^{34, 35}

In birds and reptiles, *uric acid* has the same quantitative significance in the excreta as urea has in mammals. It represents the end-product of protein metabolism in these phyla, and the amount excreted is presumably directly dependent upon the level of protein metabolism. In mammals, however, uric acid arises from the catabolism of nucleic acid and need not be discussed further in the present chapter.

Other nitrogenous constituents of urine, such as other purines and hippuric acid, will be treated elsewhere.

Absorption.—We may now return to protein taken in as food and consider its metabolic fate. It is not within the province of this chapter to discuss the digestive processes which the proteins undergo as they pass along the alimentary canal. Rather we shall start in with the absorption of the products, proceeding from the premise that normally the hydrolysis of proteins by the digestive enzymes is quite complete.* The assembly of amino acids obtained by complete hydrolysis or by mixture of individual amino acids has long been known to be an adequate substitute for protein itself.³⁷

Of undigested protein it is doubtful whether more than a trace is ever absorbed. The trace so absorbed from the alimentary canal of the infant or by the intestine of the adult under various pathologic conditions, or when certain specified proteins are eaten, may, how-

* Extensive hydrolysis of proteins, prior to absorption, was not recognized until 1909.³⁶ Until then protein digestion was thought to consist of mere solution in the intestinal juices with a slightly increased diffusibility. The radical changes in theory necessitated by the work of Kühne and Cohnheim led to equally sweeping revisions in theories of protein metabolism. Nothing remains of the Liebig theory that proteins are absorbed and assimilated without any essential change, nor of the theories of Voit and Pfleger which also postulated that the protein molecule was metabolized as a whole.

ever, be of profound importance. It is likely that some allergic conditions are referable to the absorption of undigested protein. For further information on the absorption of incompletely hydrolyzed protein the reader is referred to Cathcart's monograph.³⁸

Absorption of the amino acids takes place from the small intestine by way of the rich capillary bed of the blood vascular system. Absorption and transport by the lymph apparently does not occur.

Much of our knowledge of amino acid absorption is based upon the fundamental observations of Folin,³⁹ Van Slyke,⁴⁰ and London⁴¹ and their associates. The early failure of investigators to observe any increase in the amino acid content of the blood following a protein meal led to the theory, current until 1912, that the absorbed amino acids underwent immediate deamination in the intestinal wall or a prompt resynthesis to protein. The first of these alternatives, supported by the high ammonia values for the portal blood which Nencki and associates reported,⁴² was discredited by the work of Folin and Denis.³⁹ They were able to show that the higher ammonia content of the portal blood was due, in part, to the faulty analytical procedures then current, and partly to the action of fecal bacteria in the large intestine from which ammonia is absorbed. Van Slyke and Meyer,⁴⁰ through the development of a delicate and reliable method for the determination of amino acid nitrogen, demonstrated clearly a postprandial increase in the amino acid concentration of the blood.

In the fasting state the amino acid nitrogen content of the blood varies between 5.5 and 8 mg. per 100 cc. of blood. It rises to a maximum value of 7 to 10 mg. within four to five hours of ingesting an ordinary meal and subsides to the fasting level in about seven hours.⁴³ Studies on the absorption of individual amino acids, such as glycine and alanine, and their transitory accumulation in liver and muscle have been reported by Folin,³⁹ Van Slyke,⁴⁰ Lewis,⁴¹ Luck⁴² and their associates. Amino acids so absorbed quickly accumulate in the liver, where they may rise to a concentration, expressed as amino acid nitrogen, of 80 to 90 mg. per cent from a fasting level of 40 to 50 mg. per cent. At the same time, or later, there may be a slight increase in the amino acid content of muscle, the fasting level of which is about the same as in the liver.

Deamination.—It should be noted that the organism receives an influx of amino acids, exogenously, on absorption of the products of protein digestion; and endogenously from two main sources: As products of synthetic reactions and through hydrolysis of tissue proteins. The extent to which any given source is utilized depends, clearly, upon many factors.

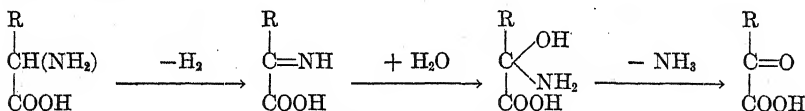
Opposed to these processes are other events and systems of reactions which tend to deplete the amino acid content of the organism. A portion of the amino acids is necessarily used for protein synthesis in connection with growth, tissue repair, and, according to some investigators, for storage. Another fraction is used in the synthesis of

related nitrogenous compounds; and still another undergoes conversion, with loss of nitrogen, into glucose, fatty acids, simple carbon compounds, and the end-products of catabolism: Carbon dioxide and water. In the course of such conversion or degradation, the nitrogen passes into the form of waste products, principally urea, and is excreted.

It is well to observe that the relative constancy in the amino acid levels of the blood and tissue fluids is only maintained by virtue of a nicely regulated balance between these opposing sets of reactions. Most of the processes mentioned are doubtless in progress simultaneously—some catabolic and others anabolic, some contributing to the amino acid store and others depleting it. The equilibrium, so-called, is obviously a very dynamic one.

The *sine qua non* of the catabolic reactions is loss of nitrogen. The process is called deamination or, by some writers, deaminization.

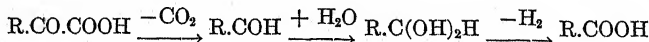
The main pathway seems to be that suggested by Knoop³⁴ and Neubauer:⁴⁶



The first reaction is catalyzed by dehydrogenases and requires the participation of a suitable hydrogen acceptor.

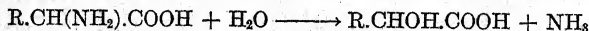
Much evidence may be advanced in support of this scheme.* In the first place, deamination by the animal organism is intimately dependent upon the presence and utilization of oxygen—a sequel to dehydrogenation.⁴⁷ The keto acids, required by theory, have also been isolated in certain cases.^{46, 47, 48} Finally, the liberation of ammonia has been demonstrated.⁴⁷ A number of amino acid oxidations, carefully studied *in vitro* by the use of such catalysts or oxidants as alloxan,⁴⁹ quinone,⁵⁰ charcoal⁵¹ and polyphenols,⁵² lend support to the same scheme.

In the presence of carboxylase, carbon dioxide is lost by the keto acid thus giving rise to an aldehyde. This, in turn, may be oxidized to the corresponding acid:



The decarboxylation is accomplished by various microorganisms and, in a few instances at least, by the higher animals. The nonenzymic deamination of amino acids *in vitro* frequently proceeds in the same way.

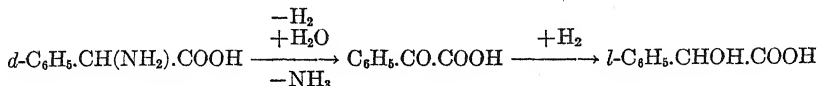
α -Hydroxyacids have also been reported as products of deamination. Their mode of formation is not definitely established. It seems unlikely that they arise directly by hydrolytic deamination:



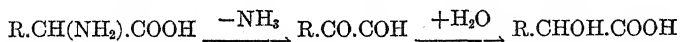
* Note its reversible character (cf. p. 627).

If so *p*-hydroxyphenyl lactic acid should form homogentisic acid in the alcaptonuric subject. Such is not the case,* despite the fact that the corresponding keto acid does so readily.^{46, 55}

It is more likely that asymmetric reduction of the keto acid is the principal means of hydroxyacid formation. As an example, we might cite the production of *l*-mandelic acid on deamination of *d*-phenyl-amino-acetic acid:^{46, 54}

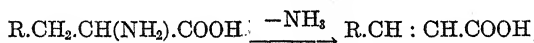


There is other evidence in support of the theory that there is a direct loss of ammonia with formation of a glyoxal



and subsequent production of a hydroxyacid on uptake of water. Actually this requires complicated rearrangements within the molecules involved but has been invoked to explain specific cases⁵⁶ and may be concerned in other instances of apparent hydrolytic deamination.^{57, 59}

Finally it is well established that deamination sometimes leads to production of an unsaturated acid in accordance with the equation:



Of the cases recorded in the literature,^{60, 61} that of aspartic acid undergoing deamination to fumaric acid has been thoroughly studied.^{61, 62} The enzyme, aspartase, which catalyzes this reaction is found in many bacteria and plants.⁶³

Deamination is accompanied by only a small reduction in free energy content. Most of the potential energy of the amino acid is made available to the organism at a later stage in metabolism.

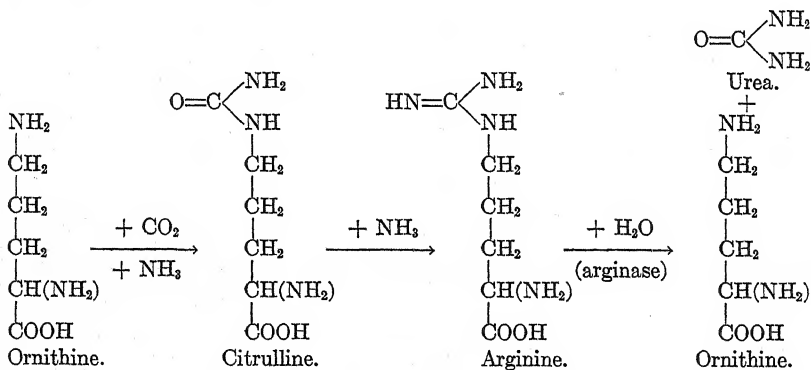
In the higher animals, deamination is centered more or less exclusively in the liver^{64, 65} according to observations made upon hepatectomized animals; but note that surviving slices of kidney tissue suspended in a suitable oxygenated medium will deaminize amino acids.⁴⁷

Urea Formation.—We may now consider the fate of the ammonia which arises in deamination. Urea is the form in which most of it will ultimately appear; and the question of immediate interest is the mechanism by which this urea is formed. No weight can be given to the once popular theory of Schmiedeberg⁶⁶ and Drechsel⁶⁷ that urea is formed from ammonium carbonate by dehydration, ammonium carbamate being an intermediate. Nor is it possible to accept, without further evidence, the cyanic-acid theory of Salkowski,⁶⁸ newly spon-

* Note, however, that phenyl lactic acid is convertible into homogentisic acid.⁵³

sored by Werner and Fearon,⁶⁹ according to which ammonium cyanate is formed and rearranges to give urea.

The recent theory of Krebs and Henseleit,⁷⁰ however, gives a satisfactory picture of the main steps and explains the salient facts. Before describing this attractive theory, it may be well to explain that the principal, if not the sole site of urea formation is the liver;⁷¹ and that the ability of the liver to form urea from ammonium salts and amino acids has been repeatedly demonstrated beginning with the classical work of Schultze and Nencki,⁷² Salkowski⁷³ and von Schroeder.⁷⁴ It should also be pointed out that arginine has long been recognized as a source of urea,⁷⁵ and that the enzyme arginase, which catalyzes the conversion, is abundant in mammalian liver.⁷⁶ Krebs and Henseleit⁷⁰ studied the formation of urea from ammonium salts by small slices of tissue in an oxygenated nutrient medium. Of 17 organs investigated, liver alone was able to form urea. The amino acids ornithine and citrulline had remarkable catalytic properties. In one experiment, 30.4 mols of extra urea per mol of ornithine were formed. The essential reactions follow:



Thus ammonia arising from deamination combines with ornithine and citrulline. Arginine is formed; and this, in the presence of arginase, rapidly hydrolyzes to urea and ornithine. The former is excreted and the latter reenters the cycle by participating anew in the formation of more arginine. The failure of birds to form much urea is probably due to the absence of arginase from the avian liver.

Urea formation from ammonia is an endothermic process:



The energy required is drawn from other oxidative reactions proceeding simultaneously.⁷⁷ This explains the need of oxygen and the maintenance of respiration for the synthesis of urea by liver tissue.

It has been already stated that urea formation by the higher animals is practically an exclusive function of the liver. This was convincingly demonstrated by Bollman, Mann, and Magath⁷¹ who showed

that hepatectomy in dogs is followed by rapid decreases in the urea content of the blood and urine.

The urea content of the blood and tissues is normally about 25–35 mg. per 100 Gm. Five to seven hours after an ordinary meal, the urea content of the blood attains a maximum of 35–50 mg. per 100 Gm.⁴⁶

The urea so formed must be regarded as a waste product. In the elasmobranchs, where very large amounts of urea are present (1700 mg. per 100 cc. of blood), there is some reason for believing that it may be of functional significance.⁷⁸ Furthermore, urea, once formed, is a stable end-product of metabolism. It passes to the kidney and is excreted unaltered.

The enzyme urease which is able to catalyze the hydrolysis of urea to ammonia and carbon dioxide is widespread in plants, especially in legumes; but in animals it occurs only in the gastric mucosa.⁷⁹ Doubtless, the high ammonia content of gastric juice is due to the activity of gastric urease. By some investigators urea is regarded as the precursor of the urinary ammonia; but the absence of urease and the recent works of Krebs⁴⁷ render this unlikely. Amino acids⁴⁷ and adenylic acid⁸⁰ are probably the substrates upon which the kidney acts in forming ammonia.

Glucogenesis.—The fate of the deamination residue—the keto acid, hydroxyacid or unsaturated acid—resulting from deamination should now be further considered.

It is evident that 21 different primary deamination products can arise by virtue of the existence of 21 different amino acids. Many of these are convertible into glucose; others may be catabolized to acetoacetic acid, β -hydroxybutyric acid and acetone; and others, doubtless, follow unknown metabolic pathways. Under normal conditions, the glucose precursors and ketone bodies which arise from the first two groups of amino acids are completely oxidized or converted into other substances useful to the organism.

The experimental evidence in support of glucogenesis from amino acids may now be presented. Much of it has been derived from studies on animals which had been rendered diabetic by pancreatectomy or injection of phlorhizin. In such animals there is a copious excretion of both glucose and urea. After a few days of starvation, when the carbohydrate reserves approach exhaustion, the glucose excreted is necessarily derived from noncarbohydrate sources. Proteins are believed to be the principal source. Assuming this to be the case, one may calculate from the relative amounts of urinary glucose and nitrogen the percentage of glucose derived from unit weight of body protein. The so-called *D/N* (dextrose/nitrogen) ratio in fasting phlorhizinized dogs is reported to be from 2.9 to 3.7⁸¹ and of a similar magnitude, possibly less, in the fasting depancreatized animal.* Lusk's school adheres to the value 3.65; from which it follows that 100 Gm. of body protein will give rise to 58 Gm. of glucose.

* For a critical treatment of the *D/N* ratio cf. Chaikoff⁸² and Macleod.⁸³

By administering known amounts of individual proteins to the fasting diabetic animal, the glucogenetic value of each may be determined from the extra glucose and extra nitrogen excreted. Thus 48 per cent of casein, 65 per cent of gelatin, and 80 per cent of gliadin are reported to be glucogenetic.⁸⁴

These differences among proteins are due to differences in amino acid distribution. By administering individual amino acids to fasting diabetic dogs, the extent to which any given acid is glucogenetic may be determined. It is necessary to know only the amounts of extra glucose and extra nitrogen excreted over and above the fasting values. The findings for all amino acids examined to date are summarized in the table on page 625.

These quantitative aspects of glucogenesis are found to be significant in the dietary treatment of various metabolic diseases accompanied by ketosis; but further discussion of ketogenesis and antiketogenesis would be foreign to the scope of the present chapter (see Chapter XXI).

Other evidence for the conversion of amino acids to carbohydrate may be found in studies on glycogen formation. It has long been known that dogs fasted for several days may be given proteins such as washed fibrin,⁸⁵ or casein⁸⁶ with a resultant deposition of liver glycogen. Of four or five amino acids which have been similarly studied by administration to rats, alanine alone has been found to increase the glycogen stores,⁸⁷ but it may be inferred that other amino acids yet to be examined will be found to act likewise.

The conditions under which certain amino acids will cause an increased concentration of glucose in the blood, or will relieve the severe hypoglycemia induced by excessive doses of insulin, have been investigated to some extent, but the results are not sufficiently uniform to justify discussion at this time.

Fat Formation.—The formation of fat from amino acids has not been extensively investigated. Mention should be made, however, of the observations of Cremer,⁸⁸ which show clearly that massive feeding of lean meat to dogs is followed by retention of carbon too large for storage in the form of glycogen.* Since carbohydrate is known to be freely convertible to fat, the glucogenetic amino acids may indirectly contribute to fat formation.

At the same time, other amino acids may participate. Reference to the table on page 625 shows that several amino acids give rise to aceto-acetic acid. This substance is in the pathway of fatty acid oxidation and by reversible processes under appropriate conditions may be condensed to fatty acids.

Thermogenic Action.—One of the curious and characteristic features of protein metabolism is the increase in metabolic rate which accompanies the ingestion of proteins. Thus, from the work of

* Note that the carbon content of fat (about 76 per cent) is much greater than the carbon content of glycogen (44 per cent).

FORMATION OF GLUCOSE* AND ACETO-ACETIC ACID FROM AMINO ACIDS†

| Substance. | Glucose formation in diabetic animal. | Aceto-acetic acid formation in perfused liver or diabetic animal. |
|-------------------------------------|---------------------------------------|---|
| Glycine..... | + | — |
| Alanine (dl-, and l-)..... | + | — |
| dl-Serine..... | + | |
| l-Cysteine..... | + | |
| l-Aspartic acid..... | + | — |
| d-Glutamic acid..... | + | — |
| β -Hydroxyglutamic acid... | + | |
| α -Amino-isobutyric acid.... | | — |
| dl-Valine..... | — | — |
| l-Leucine..... | — | + |
| dl-Isoleucine..... | — | (?) |
| Norleucine..... | | — |
| l-Proline..... | + | — |
| l-Oxyproline..... | + | |
| d-Ornithine..... | + | — |
| d-Lysine..... | — | — |
| d-Arginine..... | + | — |
| l-Histidine..... | + (?) | — (?) |
| dl-Phenylalamine..... | — | + |
| l-Tyrosine..... | — | + |
| Methionine..... | | |

* Three of the carbon atoms in each glucogenetic amino acid are convertible into glucose. In the case of glycine, which has but two carbon atoms, both are convertible.

† Cf. Dakin, H. D.¹⁸⁶

Rubner⁸⁹ and Lusk,⁹⁰ it appears that the administration of 100 Gm. of meat protein (410 Calories) to a dog would be followed within a few hours by an increase in heat output of 130 Calories.* One hundred Gm. of carbohydrate (410 Calories) would increase metabolism in the same time by only 20 Calories, and 44 Gm. of fat (410 Calories) by about 30 Calories. While it is to be understood that the ingestion of rather massive quantities of fat and carbohydrate will increase the heat

* One Calorie = 1 kilo calorie = 1000 calories.

output (metabolism of plethora), the stimulating action of protein is under all conditions much more in evidence. Probably 200 Calories of the daily output of heat by the human adult is due to this thermogenic action of protein.*

Quantitative studies have demonstrated that various proteins (beef muscle proteins, casein, gliadin, gelatin) exert effects of similar magnitude.⁹¹ Nevertheless, the individual amino acids vary greatly in thermogenic action. Lusk and his school have shown that glycine, alanine, and phenylalanine are particularly active—so much so that attempts have been made to refer the entire thermogenic action of proteins to these three amino acids.^{90, 92} But until the phenomenon has been more thoroughly studied, other amino acids may not be excluded.

Many theories have been advanced to explain the experimental observations, but none is fully satisfactory. It is clear that the increase in metabolism is not due to intestinal work ("Darmarbeit" of Zuntz), or stimuli of intestinal origin, since gum arabic, ground bones, cathartics, meat extracts, etc., are without effect. Furthermore, the response may be elicited by intravenously administered amino acids.

Rubner at one time advanced the rather picturesque hypothesis that just as the twigs of a felled tree are burned as waste so the organism chooses to burn promptly, as if useless, those amino acids which are inconvertible to glucose; thereby assuming that the glucogenetic amino acids alone are of value. The hypothesis is untenable since, *per contra*, the glucogenetic amino acids, glycine and alanine, are among the most active.⁹³

Recognition of this fact has led to the theory that possibly glucogenesis itself may be associated with exothermic reactions sufficient to account for the heat output. But if we recall that phenylalanine, which is aglucogenetic, is active thermogenically, it becomes of greater profit to consider the thermal changes theoretically possible in other reactions characteristic of amino acid catabolism. Some attempts, indeed, have been made in this direction. The free energies of various amino acids and their metabolic products are being determined and the free energy changes for various reactions of biochemical interest may be calculated.⁹⁴ Many heats of reaction are also known.† It may be shown, for example, that the degradation of glycine to urea, carbon dioxide and water is exothermic; ΔH being -158 Calories per

* Rubner⁸⁹ and Lusk⁹⁰ speak of the phenomenon as the specific dynamic action of protein.

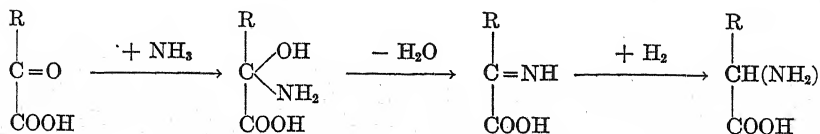
† For present purposes the heats of reaction (ΔH) are more significant than the changes in free energy (ΔF), since ΔH is a measure of the quantity of heat evolved or absorbed during the reaction. For many other purposes, especially in predicting the direction of a reaction, ΔF is more useful. Unlike ΔH it indicates whether a given reaction may take place spontaneously. Frequently ΔF and ΔH are approximately equal but cases are known in which the discrepancy is great. Sometimes the quantities are even of opposite sign. For the reactions which we are considering the free energy changes may be shown to approximate -162 , $+66$, -475 , and -132 calories, respectively.

mol of glycine. The conversion of glycine to urea and glucose [$6\text{C}_2\text{H}_5\text{NO}_2 + 3\text{CO}_2 + 3\text{H}_2\text{O} = 2\text{C}_6\text{H}_{12}\text{O}_6 + 3\text{CO}(\text{NH}_2)_2 + 3\text{O}_2$] is endothermic in character and would be accompanied by a heat absorption of 66.2 Calories per mol. The complete degradation of glutamic acid [$2\text{C}_5\text{H}_9\text{NO}_4 + 9\text{O}_2 = \text{CO}(\text{NH}_2)_2 + 9\text{CO}_2 + 7\text{H}_2\text{O}$] liberates 466 Calories per mol while glucose formation [$2\text{C}_5\text{H}_9\text{NO}_4 + 3\text{O}_2 = \text{C}_6\text{H}_{12}\text{O}_6 + \text{CO}(\text{NH}_2)_2 + 3\text{CO}_2 + \text{H}_2\text{O}$], also exothermic, would be attended by an evolution of 130 Calories per mol. It would seem reasonable to attribute part of the metabolic increase induced by amino acids to the heat liberated from these exothermic reactions; or to that excess liberated from secondary exothermic reactions, still of an unknown character, which provide the driving force for primary endothermic processes.

A portion of the heat output is associated, doubtless, with the work of excreting urea.⁹⁵ From the viewpoint of energetics, this is an inefficient process, only a portion of the energy made available for concentrating the urea being converted into useful work. The rest is given off as heat. According to Dock⁹⁶ the heat, so liberated, constitutes only a small fraction of the total thermogenic effect.

Finally, it is conceivable that certain of the amino acids or their deamination products may directly stimulate the metabolism of the entire organism or of the liver to higher levels of activity. In comparison with such dynamic agents as dinitrocresol, or 2,4-dinitrophenol, of which 20 mg. per kilo will increase the metabolic rate of an adult dog by 1000 per cent,⁹⁷ they would be very weak, but qualitatively their action may be of the same character. This hypothesis is of value in permitting a ready explanation of the specificity of amino-acid thermogenesis.

Amino Acid Synthesis.—Although the higher animals are much inferior to many micro-organisms and green plants in the matter of biosynthesis, they are still capable of synthesizing many of the amino acids. The ammonium salts of a number of keto acids when perfused through the surviving liver have been found to undergo amination to the corresponding amino acids.⁹⁸ The feeding of various "foreign" keto acids likewise leads to the formation of amino acids which, because of their foreign character, are largely excreted.⁹⁹ The mode of synthesis may, most simply, be regarded as that postulated by Knoop:³⁴



The real difficulty in amino acid synthesis apparently is not centered in these amination reactions but in synthesis of the keto acid. Thus, the inability of the organism to synthesize tryptophan is due

to its inability to synthesize β -indolepyruvic acid.¹⁰⁰ So also, histidine can be synthesized if β -imidazolpyruvic acid be provided.¹⁰¹ Both amino acids in the diet of a growing animal may be satisfactorily replaced by the corresponding keto acids.

This defect in the anabolic abilities of the higher animals, though long suspected, was first clearly revealed when attempts were made to compare the nutritive values of various food proteins. For this purpose the method of Magnus-Levy¹⁰² and Thomas¹⁰³ has been extensively used. Protein catabolism is first depressed to the minimum level by placing the animal upon a protein-free diet, of adequate calorific value. The diet is then progressively enriched by adding to it in increasing quantities the protein under investigation. The smallest quantity sufficient to restore a state of nitrogen balance is thus determined and serves, in comparison with similar values determined on other proteins, as a measure of its nutritive value. By this means gelatin, gliadin, and zein have been found to be of low nutritive value and inadequate for maintenance and growth when provided as the sole dietary protein.

We now know, by complete analysis of proteins, that such inadequacy may be correlated with the absence or relative deficiency of certain amino acids, especially tryptophan, histidine, lysine, and cystine or methionine. The existence of still another "indispensable" amino acid is rendered probable by the recent work of Rose and his associates.¹⁰⁴

It may be concluded that such observations on protein inadequacy are to be explained quite simply by considering that the higher animals are incapable of synthesizing the amino acids mentioned. This conclusion is strongly supported by the work of Osborne and Mendel¹⁰⁵ and later investigators, in which the inadequacies of various proteins were found to be corrected by addition to the diet of the missing "essential" amino acids.

So little is known about the mechanisms by which the cells elaborate their proteins from amino acids that it would be unprofitable to enter upon a discussion of this—the next and final stage in protein synthesis.

The Plasma Proteins.—The proteins synthesized by the organism are to be found in the blood, lymph, cerebrospinal fluid and all organs of the body. Those in the blood have been most extensively studied.

The principal, if not the sole proteins in the erythrocytes are hemoglobin and oxyhemoglobin. Their function in respiration, their interconvertibility at various tensions of oxygen and carbon dioxide, their origin and site of formation, and the conditions essential for synthesis are discussed elsewhere in the present volume.

As for the plasma, three proteins are known: Fibrinogen, globulin, and albumin. It is possible, if one may judge from fractionation experiments with sodium sulfate and behavior on dialysis, that the second of these is a composite of two or even three distinct globulins: Euglobulin, pseudoglobulin I, and pseudoglobulin II.¹⁰⁶ Phase rule

studies, and the work of Sørensen,¹⁰⁷ however, cast doubt upon the validity of these distinctions and indicate that these globulins are but different phases of one and the same protein.

Albumin is present in normal human plasma in a concentration of 4.5 ± 0.6 Gm. per 100 cc., globulin 2.4 ± 0.5 , and fibrinogen 0.35 ± 0.1 .

It is generally agreed that the plasma proteins originate in the liver, bone marrow, intestine or spleen. That fibrinogen is of hepatic origin seems to be definitely established, since hepatectomy causes a decrease of 30 to 40 per cent in the fibrinogen content of plasma.¹⁰⁸ In certain kinds of liver disease, such as acute yellow atrophy and following liver damage by chloroform or phosphorus, the blood shows an increase in clotting time, which is indicative of impairment in fibrinogen formation. Experiments on the site of formation of albumin and globulin have not yet yielded conclusive results.

Studies of plasma-protein formation and function have been greatly facilitated by use of the plasmapheresis technic of Abel, Rowntree and Turner.¹⁰⁹ This consists in the rapid removal of plasma, accomplished by frequent and copious withdrawals of blood with return of erythrocytes. By using this technic the maximum rate of regeneration of the plasma proteins in dogs has been found to approximate 0.4 Gm. per kilo of body weight per day.^{110, 111} In the human subject, regeneration proceeds at a maximum rate of not less than 0.15 Gm. per kilo per day¹¹⁰ and possibly much greater. The rate of plasma-protein synthesis is of importance in connection with those pathologic conditions in which there is an increased loss of plasma proteins through albuminuria or ascites.

Fibrinogen is regenerated with great rapidity, globulin less rapidly, and albumin least rapidly of the three.¹¹²

Apart from fibrinogen, which through conversion into fibrin plays an important rôle in blood clotting, the principal function of the plasma proteins is that of regulating the flow of water between the blood and tissues. Serum albumin exerts an osmotic pressure of 7.54 cm. of water per gram and serum globulin 1.95 cm. of water per gram. The total "colloid" or "oncotic" pressure of normal blood plasma is about 35 cm. The movement of water between the blood and surrounding tissues is partly determined by this osmotic pressure exerted by the plasma proteins, and partly by the filtration pressure in the capillaries. The two forces are in opposition, the former tending to draw water into the blood, the latter tending to force it out. Reduction in plasma-protein content, especially of albumin, lowers the colloid osmotic pressure and permits a flow of water through the capillary endothelium into the tissues. In hypo-albuminemia, due to plasmapheresis, edema generally appears when the albumin content falls below 1.5 per cent.¹¹³ Hypo-albuminemia, due to malnutrition or kidney disease, is likewise accompanied by edema when the serum albumin falls below a similar critical level. It follows that edema, due to vascular dis-

turbances associated with hypertension or increased capillary permeability, may also appear even though the plasma protein content is normal.

In infants and young animals the plasma-protein content is much lower than in adults.¹¹⁴ The capillary blood pressure is also lower.

Before concluding this discussion of the plasma proteins, attention should be given to the conditions under which proteins are excreted. Normally the human kidney retains the blood proteins completely; but in renal disease, especially in nephrosis, many grams may be excreted daily. The principal protein excreted is albumin, accompanied by a small proportion of globulin. The proteins are identical with those in plasma¹¹⁵ and appear in the urine, not as breakdown products of the kidney, but as unaltered constituents of plasma incompletely retained by the organ in disease.

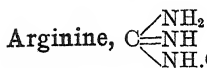
On the basis of 20,000 urines tested in the past decade, it appears that 0.6 per cent of young male adults show persistent albuminuria, of whom half have definite renal disease.¹¹⁶ Exercise, very curiously, may induce a transitory albuminuria. Thus, of 13 football players who were in the game for forty-five to sixty minutes, all exhibited albuminuria with an average urine-protein content of 2.8 per cent.¹¹⁷ All but one of the participants in the 1933 Cambridge-Oxford boat race showed albuminuria.¹¹⁸ The cause is not definitely known, although it has been attributed to increased renal permeability brought about by renal anoxemia or by the toxic action of products liberated from fatigued muscle.

In about 75 per cent of the cases of multiple myeloma an interesting protein, known as "Bence-Jones protein," is excreted. As much as 40 to 70 Gm. may be lost daily. Though heat-coagulable, the protein redissolves in the presence of salts as the temperature is elevated toward the boiling point. In some cases the protein accumulates in the plasma resulting in high values for the total plasma-protein content. The bone marrow is regarded as its site of origin.

There is little evidence of renal disease as a contributing factor in Bence-Jones proteinuria.

Rôle of the Endocrines.—As far as we now know, the glands of internal secretion exercise little control over protein metabolism. Epinephrine (adrenaline), administered to normal or adrenalectomized animals, lowers the concentration of amino acids in the blood.^{119, 120} Insulin also does so in normal animals but only through the extra epinephrine which is secreted in response to the injected insulin. It is without effect in adrenalectomized animals.¹²⁰ The full significance of this action of epinephrine is not yet established.

The Metabolism of Individual Amino Acids.—In the preceding paragraphs attention has been given to general aspects of amino acid metabolism, such as deamination, urea formation, and glucogenesis. It is now desirable to discuss in detail the metabolism of several amino acids of special interest.

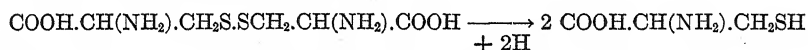


—The first step in the catabolism of arginine is its hydrolysis to ornithine and urea under the catalytic influence of arginase. Studies on the rate of urea formation from administered arginine indicate that the degradation of ornithine proceeds as rapidly as its liberation from arginine.¹⁴³ Apart from the fact that ornithine is glucogenetic in the diabetic animal, little is known about its catabolism. It should be noted that a portion of the ornithine undergoes reconversion to arginine and is thereby enabled to function catalytically in the general process of urea formation.

Since arginine is the only guanidino-compound contained in the foodstuffs, one is tempted, *a priori*, to regard arginine as the precursor of other guanidino-compounds arising as metabolic products, especially creatine. However, all attempts to demonstrate the formation of creatine from arginine or phospho-arginine have yielded only negative results.³⁵

Cystine.—The principal sources of sulfur available to the organism are the two amino acids cystine and methionine. We are, therefore, virtually obliged to regard them as the precursors of the numerous sulfur compounds with which the organism is endowed—cysteine, taurine, glutathione, ergothioneine, sulfates and sulfate esters being the simplest.

The formation of the first two of these is not difficult to picture—cysteine by reduction of cystine,



and taurine by the oxidation and decarboxylation of cysteine,



Most of the taurine in the higher animals is conjugated with cholic acid, permitting the conjecture that cysteine-cholic acid may first be formed only to be degraded subsequently to taurocholic acid. It should be observed that the cysteine-aurine conversion is based on experiments *in vitro* and awaits confirmatory evidence of a more biological nature.

Glutathione, in the reduced form a tripeptide of cysteine, glycine and glutamic acid, is probably synthesized from its constituent amino acids by the same mechanisms which lead to other peptides, although the existence of other more complicated modes of formation might be postulated.

Sulfate, free or combined, arises by oxidation of the sulfur initially present in cystine and methionine. Nothing is known of the intermediate steps. The end-product, inorganic sulfate, is partly excreted as such. The remainder undergoes conjugation with certain putrefaction products, such as phenol and indoxyl, absorbed from the large intestine. It is evident that the "total sulfate" (free plus conjugated)

content of the urine serves as a measure of the amount of cystine and methionine catabolized.

The indispensability of cystine or methionine in the diet has already been mentioned. It appears that the two substances are either interconvertible or capable of yielding, in common, some essential product of intermediary metabolism.¹²¹ The same is true of cysteine¹²² and dithioethylamine which are able to support the growth of animals on cystine-deficient diets.¹²³ *d*-Cystine cannot be used for purposes of growth in place of the naturally occurring levo enantiomorph.¹²⁴

A curious abnormality in cystine metabolism, known as "cystinuria," has long been a subject of interest. The disease is characterized by the excretion of relatively much cystine, as much as 1.8 Gm. per day, despite the rather remarkable fact that cystine administered by mouth is well catabolized.¹²⁵ The work of Brand *et al.*¹²⁶ suggests that this anomaly may be due to the excretion not of cystine but of a labile precursor of cystine. In infants the disease seems to be more severe, with a generalized deposition of cystine and pathologic involvement of various organs. It is one of the rarer types of metabolic errors. From the chemical examination of more than 10,000 urines of college students, Lewis¹²⁷ found 4 cases of acute cystinuria (crystals in the urine) and 14 cases of less severity. In the literature as a whole approximately 200 cases, mostly of the severe type (cystine calculi), have been reported to date.

An interesting familial tendency has also been demonstrated. Thus, Abderhalden¹²⁸ discovered a family in which there were 5 cystinuric children, of whom 3 died in infancy. The father and paternal grandfather were also cystinuric. Robson's case is similar.¹²⁹

The excretion of the bases putrescine and cadaverine, together with increased amounts of amino acids, has been reported occasionally. Considered in conjunction with the findings of Brand, *et al.*,¹²⁶ support is given to the theory that cystinuria is a more generalized metabolic disturbance than would be indicated by the mere excretion of increased quantities of cystine alone.

In the limited space here available, it is not possible to give an adequate treatment of many other aspects of sulfur metabolism deserving mention. The student should be aware, however, of the growing importance of this field. In oxidations and reductions, in cell proliferation, and in the action of certain enzymes, sulfhydryl compounds are believed to play a significant rôle. An economic problem of great importance has been opened up by recent discoveries that the cystine content of the diet of sheep exerts a profound effect upon the quality and yield of the wool. Several studies on the chemistry of insulin have come to focus on the sulfur-containing portion, it being indicated that much of the activity of insulin may be centered therein. For a more extended discussion of cystine, methionine, and the sulfur compounds the student should refer to the reviews by Lewis.¹³⁰

Glycine, $\text{CH}_2(\text{NH}_2).\text{COOH}$.—Being the simplest member of the α -amino acids, glycine is atypical in a number of respects. In its chemical properties it is quite anomalous and within the organism its behavior is equally distinctive. It shares with several other amino acids the property of forming glucose in the diabetic animal; but in several other respects its behavior is without parallel. Thermogenically it is one of the most active metabolic stimulants among the amino acids.⁹³ It conjugates with benzoic acid to give hippuric acid (benzoyl glycine) and in this form is one of the chief constituents of urine.

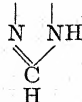
It also plays an interesting rôle in muscle physiology. This has come to light in the course of investigations on the treatment of "myasthenia gravis"—a rather rare form of muscle weakness involving the muscles of the eyes, the eyelids, and the muscles of mastication, and in its more generalized form the muscles of the arms, legs, and respiratory system. Within the past year or so, it has been shown by Boothby,¹³¹ by Remen¹³² and by Schmitt¹³³ that the continued administration of glycine in doses of 30 to 40 Gm. daily leads to remarkable clinical improvement. In view of the unfavorable prognosis hitherto attending myasthenia gravis this constitutes a significant advance.

No adequate explanation of the beneficial action of glycine can be formulated at the present time. One is tempted to postulate a defect in creatine metabolism in the myasthenic subject, partly because creatine phosphate serves as the first source of energy for muscle contraction; and partly because the myasthenic subject, unlike the normal male adult, regularly excretes creatine. The value of glycine may reside in its service as a precursor of creatine for, as a number of investigators have shown, glycine increases the creatine output in muscle dystrophies and increases the creatine content of the muscles of normal experimental animals.^{33, 134} On the other hand, progressive muscular dystrophy, in which the accompanying creatinuria is enhanced by glycine administration, shows doubtful clinical improvement in response to continued glycine therapy.^{131, 134}

It is also possible that the beneficial action of glycine in myasthenia gravis is associated with the fact that of the common amino acids, it alone diffuses freely into muscle.¹³⁵

For further elucidation of the mechanism more must be known about the therapeutic value of other amino acids [some of which are said to be creatinogenic³³], and the efficacy of creatine itself.

Histidine, $\text{HC}=\text{C}-\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$.—One of the most important



questions concerning the metabolism of histidine is the fate of the imidazole nucleus. The studies of Leiter¹³⁶ on the catabolism of histidine by dogs indicate that cleavage of the ring takes place quite

completely. On the other hand, imidazole itself and several imidazole derivatives are poorly catabolized, if at all, if one may judge from the postinjection increases in imidazole output.

The degradation of histidine is probably catalyzed by histidase,¹³⁷ an enzyme of high specificity occurring widely distributed in the livers of vertebrates. Ammonia, partly from the imidazole nucleus, is one of the products. The report that glutamic acid is also formed cannot be reconciled with the fact that histidine is not glucogenetic in the diabetic animal, a property which it should possess if glutamic acid is formed.¹³⁸

The intermediate products of histidine catabolism appear to be imidazole pyruvic acid, imidazole lactic acid, and possibly imidazole acrylic acid (urocanic acid). The last-named substance was first reported as a constituent of dogs' urine, especially of animals to which large doses of histidine had been administered.¹³⁹

The normal excretion of imidazole bodies by man is from 150 to 600 mg. per day¹⁴⁰ and is increased on ingestion of proteins. In diseases of the hepatic parenchyma the daily output rises to 800 to 1000 mg.

Histamine, derived from histidine by decarboxylation, is an amine of great pharmacological activity. In view of the probability that small amounts of histamine may be absorbed from the large intestine as a product of bacterial action, it is of interest that an enzyme, histaminase, has been discovered which catalyzes its degradation.¹⁴¹ The enzyme is most abundant in the kidney and intestine.

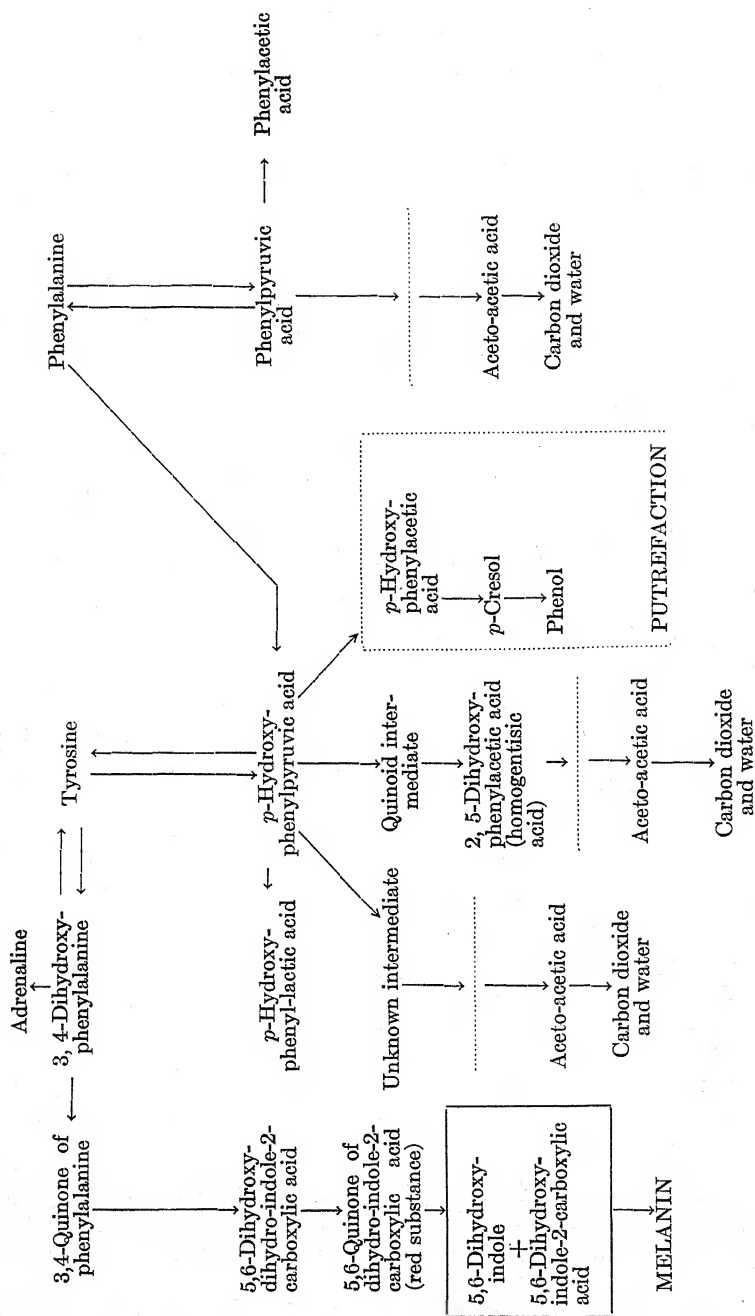
Histidine has also been a source of interest to students of nutrition, in that it constitutes one of the indispensable amino acids and serves furthermore as a precursor of purines.¹⁴²

The student should notice the relationship of histidine to ergothioneine (thiohistidine betaine) and carnosine (a dipeptide of histidine and β -alanine). The first of these substances, long known as a base in ergot, was rediscovered in 1925 in red blood cells. Carnosine and anserine are constituents of muscle. Little is known about their metabolism.

Phenylalanine and Tyrosine.—Since these two amino acids are closely related, both in structure and in metabolism, it is desirable that they be considered together. Their fate in the body may be summarized as in chart on page 635.

The end-products of their metabolism are carbon dioxide and water with the probable formation of aceto-acetic acid as an intermediate.

Part of the evidence upon which this scheme is based arises from the study of an interesting and peculiar error in metabolism called "alcaptonuria," first described by v. Boedeker in 1861.¹⁴⁴ The disease, if it may be so called, is rare—not more than 50 or 60 cases having been reported. It occurs in subjects of all ages and persists from infancy to old age with no associated ill effects. Apparently, the well-being of the subject is unimpaired. Occasionally in middle life ochro-



nosis develops. The cartilaginous tissues become blackened just as in the case of persons who over long periods have applied carbolic acid dressings to ulcerated areas.

The urine of the alcaptonuric subject rapidly blackens on exposure to air, due to the oxidation of a labile substance known as *homogentisic acid*. This compound, first discovered by Marshall,¹⁴⁵ was extensively studied by Wolkow and Baumann¹⁴⁶ and shown to be 2,5-dihydroxyphenylacetic acid. It arises both from tyrosine and phenylalanine,¹⁴⁷ since the ingestion of either substance by the alcaptonuric subject increases the excretion of homogentisic acid. Homogentisic acid, if it be administered, is excreted unaltered. In the normal subject it is fully oxidized, while if perfused through a surviving liver it yields aceto-acetic acid.¹⁴⁸ This suggests that homogentisic acid arises normally in the catabolism of both phenylalanine and tyrosine and that the defect observed in alcaptonuria is merely a blocking at the homogentisic acid stage. Whether or not the metabolism of tyrosine and phenylalanine is exclusively restricted to the central pathway shown is not fully established. Since Dakin has shown that *p*-methylphenylalanine and *p*-methoxyphenylalanine, which cannot form quinonoid derivatives, are fully catabolized by the alcaptonuric subject,¹⁴⁹ it would seem that aceto-acetic acid may form by an alternative set of reactions not involving any quinonoid intermediate. Such a supplementary pathway is included in the scheme. According to Dakin, alcaptonuria is characterized both by failure to catabolize homogentisic acid and by an enhanced formation of the quinonoid intermediate from which it is derived.

The formation of *p*-hydroxyphenylpyruvic acid from phenylalanine is to be inferred from the work of Kotake and associates¹⁵⁰ and from observations by Medes on a curious metabolic error known as "tyrosinosis."¹⁵¹ In this abnormal condition, of which only one case has been discovered, relatively much *p*-hydroxyphenylpyruvic acid is excreted. Elevation of tyrosine metabolism by administration of the amino acid is followed by the excretion of, first, tyrosine, later, *l*-*p*-hydroxyphenyl-lactic acid, and finally, as the metabolism is raised still further, *l*-3,4-dihydroxyphenylalanine. The last two compounds are thought to arise through side reactions. In addition to tyrosine, either phenylalanine or ingested protein is able to increase the subject's output of *p*-hydroxyphenylpyruvic acid. The metabolic error seems to consist in a retardation in the first stages of tyrosine metabolism and inability to oxidize a *p*-hydroxy metabolite in the 2,5-position.

In addition to forming *p*-hydroxyphenylpyruvic acid, phenylalanine undergoes oxidative deamination to phenylpyruvic acid.¹⁵² The latter may undergo a partial and irreversible conversion to phenylacetic acid but, presumably, the greater portion passes on to aceto-acetic acid, carbon dioxide, and water. Nothing is definitely established about the mechanism of ring cleavage leading to aceto-acetic acid formation.

Still another avenue is open by which tyrosine may be catabolized.

In the presence of tyrosinase, tyrosine is oxidized to the brownish-black pigment *melanin*, which occurs normally in the retina, skin, and hair of the higher animals, the feathers of birds, the epidermal pigments of many insects, and the "ink" of cephalopods. "Sun tan" is associated with increased melanin formation. Albinism is partly characterized by a failure of melanin formation. Pathologically, an extensive deposition of melanin, sometimes accompanied by melanuria, takes place in melanotic tumors.

The mode of formation of melanin as proposed by Raper¹⁵³ is included in the scheme presented above. Note that melanin itself is thought to be a polymer or condensation product of many indole molecules having the empirical formula $(C_5H_5O_3N)_n$ or $(C_8H_7O_3N)$.

In the large intestine, through the action of putrefactive bacteria, *p*-cresol and phenol may be formed.

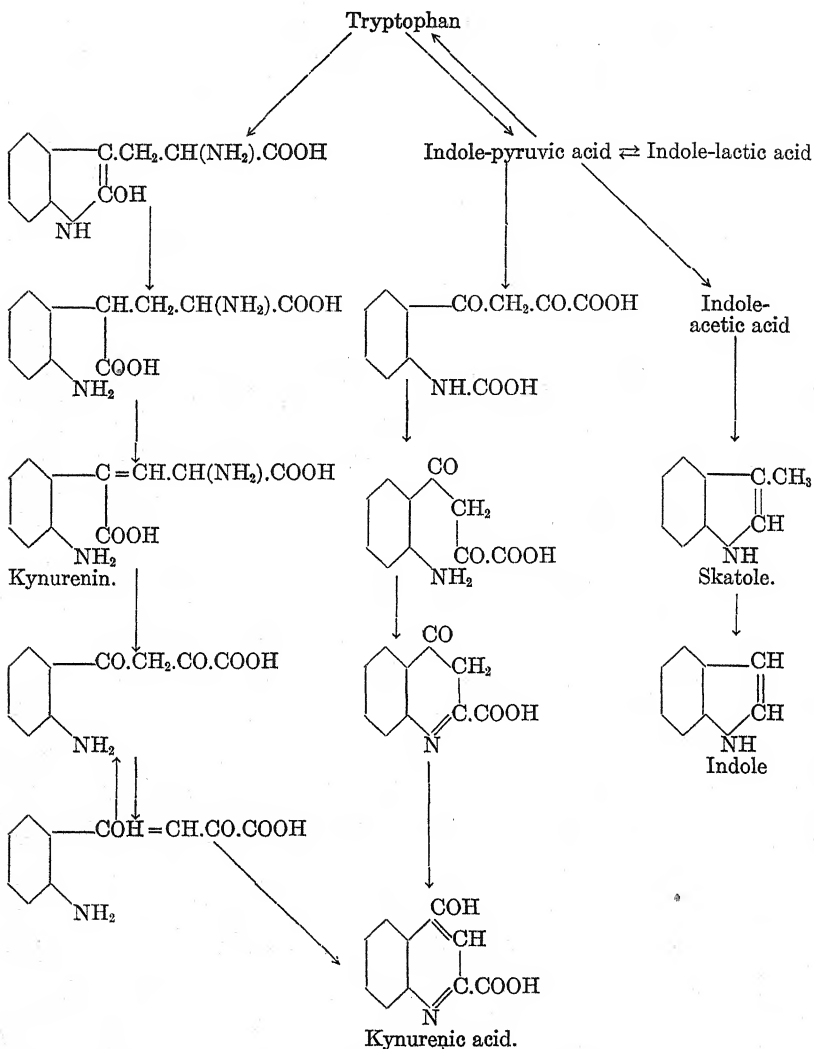
Tryptophan.—Ever since its isolation by Hopkins and Cole in 1901,¹⁵⁴ tryptophan has been one of the most actively investigated of the amino acids. Its importance is partly due to its indispensability in the nutrition of the higher animals, partly to the unique nature of its metabolism and partly to a number of special rôles of physiologic significance assigned to it from time to time. Thus it has been shown on ingestion to increase the urochrome fraction of the urine¹⁵⁵ and may therefore be regarded as a precursor of the urinary pigment. It is also said to promote hemoglobin formation both in normal animals¹⁵⁶ and in certain human anemias.¹⁵⁷ But before full weight can be given to such observations, it is necessary that they receive adequate confirmation by other investigators.

The course of tryptophan catabolism may be represented schematically (p. 638).

The scheme, which includes indole-pyruvic acid, was proposed by Ellinger and Matsuoka¹⁵⁸ following upon the discovery of kynurenic acid. The alternative pathway is rendered necessary by work of recent date which shows that tryptophan gives rise to kynurenin, itself an intermediate in kynurenic acid formation.¹⁵⁹ No single metabolic pathway can be devised which will give due recognition to both indole-pyruvic acid and kynurenin. The existence of alternative pathways is therefore clearly indicated; and, indeed, there is good reason for believing that many metabolites may be synthesized or degraded along a variety of routes.

That indole-pyruvic acid may be formed in the course of tryptophan metabolism is rendered probable by analogy with other amino acids, and by its ready conversion into kynurenic acid in the rabbit. The reversible character of its formation is proven by the fact that indole-pyruvic acid will satisfactorily replace tryptophan as a dietary supplement in animal nutrition. The place of indole-lactic acid is not firmly established; but provisionally it may be considered to arise from indole-pyruvic acid by reduction.

It is well to observe that kynurenic acid is further catabolized by



man and certain animals. Indeed, even in the rabbit, in which species most of the work upon kynurenin and kynurenic acid has been done, it is unlikely that kynurenic acid is entirely resistant to ring cleavage and oxidation. The formation of kynurenin from tryptophan is irreversible, since kynurenin added to tryptophan-deficient diets in place of tryptophan does not permit satisfactory growth or maintenance.¹⁶⁰

It is of passing interest that both *d*- and *l*-tryptophan may be utilized by rats, though it is generally true that foreign enantiomorphs are physiologically inert. Thus *d*-cystine is not able to replace *l*-cystine in animal nutrition.

Tryptophan is also of interest in being the precursor of indoxyl-

sulfuric acid and indican which have long been known as occasional excretory products of the higher animals.

Clinical Applications.—Thus far attention has been centered, primarily, upon the metabolism of proteins and amino acids in the normal organism. It may be well to consider several pathologic conditions in which the amino acids and their immediate metabolites suffer departures from the normal in distribution, excretion or utilization.

In chronic glomerular nephritis (nephrotic type) and in amyloid nephrosis, both the total protein content and serum albumin are low. An obstinate type of edema, unaccompanied by cardiac disorders or significant vascular disturbances, is found to develop when the serum protein content falls below 4 per cent.¹⁶¹ The albumin deficiency is largely due to massive albuminuria. Low serum albumin is regarded more and more as the cause of nephrotic edema.

A deficiency in serum albumin is also observed in acute nephritis and frequently in heart failure. In such cases evidence of malnutrition, more specifically of protein starvation arising from long-continued dietary deficiencies, or from inadequate synthesis of the serum proteins, is common. To this the serum-albumin deficiency may be referred. The attendant edema is primarily due to hypertension, increased capillary permeability and, in heart failure, increased capillary blood pressure due to venous congestion.

There is more and more of a tendency to emphasize malnutrition (protein starvation) as a characteristic symptom, to recognize nephritis as a wasting disease, and to direct the treatment, in part, toward restoring the serum-albumin content to a normal level. This necessitates, in many cases, the abandonment of low-protein diets and the maintenance of the protein intake at a fairly high level.¹⁶²

Numerous cases of edema, endemic in form, nutritional in origin, and referable to low serum albumin have been reported from time to time.¹⁶³ In tuberculosis, diabetes, and gastro-intestinal disease lowered serum albumin is commonly observed. In such cases, the evidence of malnutrition as the causal agent is quite clear, since there is no renal, cardiac, or vascular involvement; and restoration of the serum albumin to the normal level gradually follows upon protein enrichment of the diet.¹⁶¹

It should be noted that hypo-albuminemia (low serum albumin) is the most common departure from the normal in serum-protein content in the diseases mentioned. The serum-globulin content usually remains unaltered or may even increase slightly, coincident with a decrease in serum albumin. In infantile eczema, however, an increase in serum albumin and a decrease in globulin have been observed.¹⁶⁴

Serum-albumin determinations are usually of greater significance than total serum proteins. Albumin/globulin ratios are of doubtful value.

Since the monumental work of Fåhræus,¹⁶⁵ the sedimentation rate of the erythrocytes in citrated blood has come to be of clinical value.

In active tuberculosis, arthritis, febrile infections and inflammatory conditions generally, the erythrocytes are found to sediment out more rapidly than in normal blood. This is largely due to increased agglutination of the erythrocytes brought about, in turn, by increases in fibrinogen and globulin content. Normally, the sedimentation rate increases in pregnancy, is always slightly higher in women than in men, and increases progressively with advance in age. Clinically the test is of little specific diagnostic value, but it is a useful aid in therapy. Thus, a continued high sedimentation rate in a tubercular subject, though all other clinical signs have disappeared in response to treatment, is regarded as evidence of an active lesion.

Determinations of the amino nitrogen content of blood have but little clinical significance. The values are abnormally high in acute yellow atrophy of the liver, in myeloid leukemia and in polycythemia; but in all other pathologic conditions hitherto studied, the deviations from the normal are inappreciable.

It is likewise true that determinations of the amino nitrogen content of the urine have not yet been found to be of clinical value. It should be observed, however, that the inborn errors of metabolism—cystinuria and alcaptonuria—involving specific amino acids, are revealed through urine analysis. In certain severe forms of liver disease, especially in acute yellow atrophy, there is an enhanced excretion of amino acids. This is indicated by crystalline deposits of the less soluble amino acids, cystine, leucine, and tyrosine, which have been known to investigators for many years. A marked increase in the excretion of imidazole bodies has been reported in diseases of the hepatic parenchyma.¹⁴⁰

The therapeutic value of one of the amino acids, glycine, in the treatment of myasthenia gravis has already been mentioned (p. 633).

Of the metabolic products of amino acids, urea has been found to be of considerable interest and value in the diagnosis of renal disease. The normal kidney excretes urea with such efficiency that the urea content of blood, which in the fasting state approximates 20–35 mg. per 100 cc., seldom increases above 50 mg. per 100 cc. following the digestion of an ordinary meal. Values above 50 mg. per 100 cc. indicate an impairment in the ability of the kidney to excrete urea and are indicative of renal disease. The rate at which the kidney, under defined conditions, excretes administered urea has been proved to be an even more valuable means of testing renal function.

In acute intestinal obstruction the urea content of the blood may also be elevated.*

J. MURRAY LUCK.

* Of necessity, the topics discussed in the preceding paragraphs have been treated with great brevity. If a more extended presentation of specific subjects is desired reference to the papers of original publication is, of course, advisable. The reviews on protein and amino acid metabolism appearing from year to year in the Annual Review of Biochemistry are valuable sources of reference to the advanced student. Two or three monographs may also be recommended.¹⁶⁶ Clinical aspects are thoroughly treated by Peters and Van Slyke in their excellent two-volume work.¹⁶⁷

REFERENCES

1. Vickery, H. B., and Schmidt, C. L. A.: *Chem. Rev.*, **9**, 169 (1931).
2. Gay-Lussac and Thenard: *Ann. chim.* (First series), **74**, 47 (1810); *J. phys. chim. hist. nat. arts*, **70**, 257 (1810).
3. Liebig, J.: *Animal Chemistry* (Published by John Owen, 2nd Edition, 1843), p. 21.
4. Atwater, W. O.: *U. S. Dept. Agr., Farmers' Bull.*, **142**, (1902).
5. McLellan, W. S., and Hannon, R. R.: *J. Biol. Chem.*, **95**, 327 (1932).
6. Sherman, H. C.: *J. Biol. Chem.*, **41**, 97 (1920).
7. Susskind, B.: *Arch. Verdauungs-Krankh.*, **52**, 74 (1932); Rubner, M.: *Z. ges. expil. Med.*, **72**, 123 (1930).
8. Voit, E.: *Z. Biol.*, **93**, 15 (1932).
9. Robison, R.: *Biochem. J.*, **16**, 131 (1922).
10. Smith, M.: *J. Biol. Chem.*, **68**, 15 (1926).
11. Deuel, H. J., Jr., Sandiford, I., Sandiford, K., and Boothby, W. M.: *J. Biol. Chem.*, **76**, 391 (1928).
12. Voit, C.: Hermann's *Handbuch der Physiologie* (1881), **6**, p. 300.
13. Pfüger, E.: *Arch. ges. Physiol.*, **54**, 333 (1893).
14. Voit, C.: Hermann's *Handbuch der Physiologie* (Leipzig, 1881), **6**, p. 518.
15. Woods, C. D., and Mansfield, E. R.: *U. S. Dept. Agr. Exp. Station Bull.*, **149** (1904).
16. Krogh, A., and Krogh, M.: *A Study of the Diet and Metabolism of Eskimos* (1913); Lusk, G.: *Science of Nutrition* (1928), p. 457.
17. McCay, D.: *Scientific Memoirs Officers Med. San. Depts. Govt. India*, **34** (1908); **48** (1911).
18. Campbell, J. A.: *Biochem. J.*, **13**, 239 (1919).
19. Chittenden, R. H.: *Physiological Economy in Nutrition* (1904); *The Nutrition of Man* (1907).
20. Rubner, M.: *Münch. med. Wochschr.*, Suppl. to No. 8 (1918); cited by Lusk.²¹
21. Lusk, G.: *Physiol. Rev.*, **1**, 523 (1921).
22. Folin, O.: *Am. J. Physiol.*, **13**, 45 (1905).
23. Fourcroy, A. F., and Vauquelin, L. N.: *Ann. chim. phys.*, **31**, 48 (1799).
24. Rouelle, H. M.: *J. médecine* (1773).
25. Boerhaave, J.: *A New Method of Chemistry* (1727).
26. Berzelius, J.: *J. Chem. Phys.* (Schweigger), **11**, 261 (1814).
27. Boussingault: *Ann. chim. phys.* III, **29**, 472 (1850).
28. Folin, O.: *Am. J. Physiol.*, **13**, 66 (1905).
29. Sullivan, M. X., and Hess, W. C.: *J. Biol. Chem.*, **102**, 67 (1933).
30. Hunter, A.: *Creatine and Creatinine* (1928).
31. Rose, W. C., and Dimmitt, F. W.: *J. Biol. Chem.*, **26**, 345 (1916).
32. Benedict, S. R., and Osterberg, E.: *J. Biol. Chem.*, **56**, 229 (1923).
33. Beard, H. H., and Barnes, B. O.: *J. Biol. Chem.*, **94**, 49 (1931).
34. Knoop, F.: *Z. physiol. Chem.*, **67**, 489 (1910).
35. Hyde, E. C., and Rose, W. C.: *J. Biol. Chem.*, **84**, 535 (1929); Grant, R. L., Christman, A. A., and Lewis, H. B.: *Proc. Soc. Exptl. Biol. Med.*, **27**, 231 (1929); Brown, D. M., and Luck, J. M.: *Proc. Soc. Exptl. Biol. Med.*, **29**, 723 (1932).
36. Cohnheim, O.: *Z. physiol. Chem.*, **33**, 451 (1901); **49**, 64 (1906); **51**, 415 (1907); **59**, 239 (1909).
37. Abderhalden, E.: *Z. physiol. Chem.*, **77**, 22 (1912); Loewi, O.: *Arch. exptl. Path. Pharmacol.*, **48**, 303 (1902).
38. Cathcart, E. P.: *The Physiology of Protein Metabolism* (1921).
39. Folin, O., and Denis, W.: *J. Biol. Chem.*, **11**, 87, 161; **12**, 141, 253 (1912).
40. Van Slyke, D. D., and Meyer, G. M.: *J. Biol. Chem.*, **12**, 399; **16**, 197, 213 (1913).
41. London, E. S.: *Z. physiol. Chem.* A long series of papers including especially **53**, 246 (1907); **56**, 378 (1908); **60**, 194, 267, 274 (1909); **61**, 69 (1909).
42. Nencki, M., Pawlow, J. P., and Zaleski, J.: *Arch. exptl. Path. Pharmacol.*, **37**, 26 (1896).
43. Folin, O., and Berglund, H.: *J. Biol. Chem.*, **51**, 395 (1922).
44. Wilson, R. H., and Lewis, H. B.: *J. Biol. Chem.*, **84**, 511 (1929).
45. Luck, J. M.: *J. Biol. Chem.*, **77**, 13 (1928); Luck, J. M., and Seth, T. N.: *Biochem. J.*, **19**, 366 (1925).
46. Neubauer, O.: *Deut. Arch. klin. Med.*, **95**, 211 (1909); *Handb. norm. path. Physiol.* (Bethe), **5**, 671 (1928).

47. Krebs, H. A.: *Z. physiol. Chem.*, **217**, 191; **218**, 157 (1933).
48. Kotake, Y., et al., *Z. physiol. Chem.*, **122**, 201, 206, 211, 220 (1922).
49. Strecker, A.: *Ann.*, **123**, 363 (1862).
50. Traube, W.: *Ber.*, **44**, 3145 (1911).
51. Warburg, O.: *Biochem. Z.*, **119**, 134 (1921); Wieland, H., and Bergel, F.: *Ann.*, **439**, 196 (1924).
52. Happold, F. C., and Raper, H. S.: *Biochem. J.*, **19**, 92 (1925); Robinson, M. E., and McCance, R. A.: *Biochem. J.*, **19**, 251 (1925).
53. Neubauer, O., and Falta, W.: *Z. physiol. Chem.*, **42**, 81 (1904).
54. Neubauer, O., and Fischer, H.: *Z. physiol. Chem.*, **67**, 230 (1910).
55. Neubauer, O., and Gross, W.: *Z. physiol. Chem.*, **67**, 219 (1910).
56. Dakin, H. D., and Dudley, H. W.: *J. Biol. Chem.*, **15**, 127 (1913).
57. Ehrlich, F., and Jacobsen, K. A.: *Ber.*, **44**, 888 (1911).
58. Kotake, Y., et al., *Z. physiol. Chem.*, **143**, 218 (1925).
59. Embden, G., and Baldes, K.: *Biochem. Z.*, **55**, 301 (1913).
60. Raistrick, H.: *Biochem. J.*, **11**, 71 (1917); Hirai, K.: *Biochem. Z.*, **114**, 67 (1921).
61. Quastel, J., and Woolf, B.: *Biochem. J.*, **20**, 545 (1926).
62. Borsook, H., and Huffman, H. M.: *J. Biol. Chem.*, **99**, 663 (1933).
63. Virtanen, A. I., and Tarnanen, J.: *Biochem. Z.*, **250**, 193 (1932); Ishihara, T.: *Chem. Abstracts*, **26**, 3539 (1932).
64. Bollman, J. L., Mann, F. C., and Magath, T. B.: *Am. J. Physiol.*, **78**, 258 (1926).
65. Bollman, J. L., and Mann, F. C.: *Am. J. Physiol.*, **92**, 92 (1930).
66. Schmiedeberg, O.: *Arch. exper. Path. Pharmacol.*, **8**, 1 (1878).
67. Drechsel, E.: *J. prakt. Chem.* (2), **22**, 481 (1880); *Ber.*, **23**, 3096 (1890).
68. Salkowski, E.: *Z. physiol. Chem.*, **1**, 1 (1877); **4**, 54, 100 (1880); cf. also Hofmeister, F.: *Arch. exper. Path. Pharmacol.*, **37**, 426 (1896).
69. Werner, E. A.: *The Chemistry of Urea* (1923); Fearon, W. R.: *Physiol. Rev.*, **6**, 399 (1926).
70. Krebs, H. A., and Henseleit, K.: *Z. physiol. Chem.*, **210**, 33 (1932).
71. Bollman, J. L., Mann, F. C., and Magath, T. B.: *Am. J. Physiol.*, **69**, 371 (1924).
72. Schultzen, O., and Nencki, M.: *Z. Biol.*, **8**, 124 (1872).
73. Salkowski, E.: *Ber.*, **8**, 116 (1875); *Z. physiol. Chem.*, **1**, 1 (1877).
74. v. Schroeder, W.: *Arch. exper. Path. Pharmacol.*, **15**, 364 (1882).
75. Kossel, A., and Dakin, H. D.: *Z. physiol. Chem.*, **41**, 321; **42**, 181 (1904).
76. Hunter, A., and Dauphinee, J. A.: *Proc. Roy. Soc. (London)*, **97B**, 227 (1925).
77. Löffler, W.: *Biochem. Z.*, **112**, 164 (1920); Borsook, H., and Keighley, G.: *Proc. Natl. Acad. Sci.*, **19**, 626, 720 (1933).
78. Simpson, W. W., and Ogden, E.: *J. Exper. Biol.*, **9**, 1 (1932).
79. Luck, J. M.: *Biochem. J.*, **18**, 825 (1924).
80. Embden, G., et al., *Arch. ges. Physiol.*, **223**, 487 (1930); *Z. physiol. Chem.*, **190**, 62 (1930).
81. Rapport, D.: *Physiol. Rev.*, **10**, 349 (1930).
82. Chaikoff, I. L.: *J. Biol. Chem.*, **74**, 203 (1927).
83. Macleod, J. J. R.: *The Fuel of Life* (1928).
84. Janney, N. W.: *J. Biol. Chem.*, **20**, 321 (1915).
85. Bernard, C.: *Leçons sur la Diabète* (1877).
86. Rosenfeld, G.: *Biochem. Z.*, **42**, 403 (1912).
87. Wilson, R. H., and Lewis, H. B.: *J. Biol. Chem.*, **85**, 559 (1930).
88. Cremer, M.: *Münch. med. Wochschr.*, **44**, 811 (1897); *Z. Biol.*, **38**, 309 (1899).
89. Rubner, M.: *Die Gesetze des Energieverbrauches bei der Ernährung* (1902).
90. Lusk, G.: *The Science of Nutrition* (1928).
91. Rapport, D.: *J. Biol. Chem.*, **60**, 497 (1924).
92. Rapport, D., and Beard, H. H.: *J. Biol. Chem.*, **73**, 299 (1927).
93. Lusk, G.: *J. Biol. Chem.*, **13**, 155 (1912-13).
94. Parks, G. S., and Huffman, H. M.: *Free Energies of Some Organic Compounds* (1932); Borsook, H., and Huffman, H. M.: *J. Am. Chem. Soc.*, **54**, 4297 (1932); *J. Biol. Chem.*, **99**, 663 (1933); Greenstein, J. P.: *J. Biol. Chem.*, **101**, 603 (1933).
95. Borsook, H., and Winegarden, H. M.: *Proc. Natl. Acad. Sci.*, **17**, 3, 13, 75 (1931).

96. Dock, W.: *Am. J. Physiol.*, 106, 745 (1933).
97. Hall, V. E., Field, J., Sahyun, M., Cutting, W. C., and Tainter, M. L.: *Am. J. Physiol.*, 106, 432 (1933).
98. Embden, G., and Schmitz, E.: *Biochem. Z.*, 29, 423 (1910); 38, 393 (1912).
99. Kondo, K.: *Biochem. Z.*, 38, 407 (1912).
100. Berg, C. P., Rose, W. C., and Marvel, C. S.: *J. Biol. Chem.*, 85, 219 (1930).
101. Harrow, B., and Sherwin, C. P.: *J. Biol. Chem.*, 70, 683 (1926).
102. Magnus-Levy, A.: *Von Noorden's Metabolism and Practical Medicine*, Vol. i (English edition), p. 72 (1907).
103. Thomas, K.: *Arch. Anat. Physiol.*, 219 (1909).
104. Rose, W. C., et al., *J. Biol. Chem.*, 94, 155, 167, 173 (1931).
105. Osborne, T. B., and Mendel, L. B.: *J. Biol. Chem.*, 12, 81 (1912); 15, 311 (1913); 16, 423 (1913); 17, 325 (1914).
106. Howe, P. E.: *Physiol. Rev.*, 5, 439 (1925).
107. Sørensen, S. P. L.: *Kolloid-Z.*, 53, 102, 170, 306 (1930).
108. Jones, T. P., and Smith, H. P.: *Am. J. Physiol.*, 94, 144 (1930).
109. Abel, J. J., Rowntree, L. G., and Turner, B. B.: *J. Pharmacol.*, 5, 625 (1914).
110. Barnett, C. W., Jones, R. B., and Cohn, R. B.: *J. Exptl. Med.*, 55, 683 (1932).
111. Whipple, G. H., et al., *Am. J. Physiol.*, 47, 356 (1918); 52, 54 (1920).
112. Barker, M. H., and Kirk, E. J.: *Arch. Internal Med.*, 45, 319 (1930); Leiter, L.: *Arch. Internal Med.*, 48, 1 (1931); Kumpf, A. E.: *Arch. Path.*, 13, 415 (1932); Lepore, M. J.: *Arch. Internal Med.*, 50, 488 (1932).
113. Darrow, D. C., et al., *J. Clin. Investigation*, 11, 683, 701 (1932).
114. Clark, G. A., and Holling, H. E.: *J. Physiol.*, 73, 305 (1931); Swanson, P. P., and Smith, A. H.: *J. Biol. Chem.*, 97, 745 (1932).
115. Widdowson, E. M.: *Biochem. J.*, 27, 1321 (1933).
116. Diehl, H. S., and McKinlay, C. A.: *Arch. Internal Med.*, 49, 45 (1932).
117. Dill, D. B., et al., *Am. J. Physiol.*, 98, 352 (1931).
118. Thornton, J. W., and White, E. G.: *J. Physiol.*, 78, 23P (1933).
119. Luck, J. M., and Morse, S. W.: *Biochem. J.*, 27, 1648 (1933).
120. Davis, B. L., and Van Winkle, W.: *J. Biol. Chem.*, 104, 207 (1934).
121. Jackson, R. W., and Block, R. J.: *J. Biol. Chem.*, 98, 465 (1932); Weichselbaum, T. E., et al., *Nature*, 129, 795 (1932); White, A., and Lewis, H. B.: *J. Biol. Chem.*, 98, 607 (1932).
122. Mitchell, H. H.: *J. Nutrition*, 4, 95 (1931).
123. Sullivan, M. X., et al., *U. S. Pub. Health Service. Suppl. Pub. Health Repts.*, 89, 16 pp (1931).
124. du Vigneaud, V., et al., *J. Biol. Chem.*, 98, 577 (1932).
125. Magnus-Levy, A.: *Biochem. Z.*, 156, 150 (1925).
126. Brand, E., et al., *J. Biol. Chem.*, 86, 315 (1930).
127. Lewis, H. B.: *Ann. Internal Med.*, 6, 183 (1932).
128. Abderhalden, E.: *Z. physiol. Chem.*, 38, 557 (1903).
129. Robson, W.: *Biochem. J.*, 23, 138 (1929).
130. Lewis, H. B.: *Ann. Rev. Biochem.*, 1, 172 (1932); 2, 95 (1933); 4 (1935); *Physiol. Rev.*, 4, 394 (1924); *Yale J. Biol. Med.*, 4, 437 (1932).
131. Boothby, W. M.: *Proc. Staff Meetings Mayo Clinic*, 7, 557, 737 (1932); 9, 593 (1934).
132. Remen, L.: *Deut. Z. Nerv.*, 128, 66 (1932).
133. Schmitt, E. O. G.: *Ann. Internal Med.*, 7, 948 (1934).
134. Brand, E., et al., *J. Biol. Chem.*, 92, lix (1932); *J. Biol. Chem.*, 87, ix (1930); *Am. J. Physiol.*, 90, 296 (1929); Thomas, K., et al., *Z. physiol. Chem.*, 205, 93 (1932); Milhorat, A. T.: *Deut. Arch. klin. Med.*, 174, 487 (1933); Kostakow, S., and Slauck, A.: *Deut. Arch. klin. Med.*, 175, 25 (1933).
135. Luck, J. M.: *J. Biol. Chem.*, 77, 13 (1928).
136. Leiter, L.: *J. Biol. Chem.*, 64, 125 (1925).
137. Abderhalden, E., and Buadze, S.: *Z. physiol. Chem.*, 200, 87 (1931); Kauffman, F., and Mislowitzer, E.: *Biochem. Z.*, 226, 325 (1930); Edlbacher, S., and Kraus, J.: *Z. physiol. Chem.*, 195, 267 (1931).
138. Edlbacher, S., and Kraus, J.: *Z. physiol. Chem.*, 191, 225 (1930).
139. Jaffe, M.: *Ber.*, 7, 1669 (1874); 8, 811 (1875); Kotake, Y., and Konishi, M.: *Z. physiol. Chem.*, 122, 230 (1920).
140. Kauffman, F., and Engel, R.: *Z. klin. Med.*, 114, 405 (1930).
141. Best, C. H., and McHenry, E. W.: *J. Physiol.*, 70, 349 (1930).

142. Ackroyd, H., and Hopkins, F. G.: *Biochem. J.*, **10**, 551 (1916).
143. Kiech, V. C., Luck, J. M., and Smith, A. E.: *J. Biol. Chem.*, **90**, 677 (1931).
144. v. Boedecker, C.: *Ann.*, **117**, 98 (1861).
145. Marshall, J.: *Am. J. Pharm.*, **59**, 131 (1887).
146. Wolkow, M., and Baumann, E.: *Z. physiol. Chem.*, **15**, 228 (1891).
147. Falta, W., and Langstein, L.: *Z. physiol. Chem.*, **37**, 513 (1902).
148. Embden, G., and Baldes, K.: *Biochem. Z.*, **55**, 301 (1913).
149. Dakin, H. D.: *J. Biol. Chem.*, **9**, 151 (1914).
150. Kotake, Y., et al., *Z. physiol. Chem.*, **122**, 195 (1922).
151. Medes, G.: *Biochem. J.*, **26**, 917 (1932).
152. Chandler, J. P., and Lewis, H. B.: *J. Biol. Chem.*, **96**, 619 (1932); Lewis, H. B., et al., *J. Biol. Chem.*, **92**, 499 (1931).
153. Raper, H. S.: *Biochem. J.*, **20**, 735 (1926); **21**, 89 (1927).
154. Hopkins, F. G., and Cole, S. W.: *J. Physiol.*, **27**, 418 (1901); **29**, 451 (1903).
155. Tani, Y.: *Mitt. med. Ges. Osaka*, **24**, 1457 (1925); Kotake, Y., and Sakata, H.: *Z. physiol. Chem.*, **195**, 184 (1931).
156. Fontès, G., and Thivolle, L.: *Compt. rend.*, **191**, 1088 (1930); *Sang*, **4**, 658 (1930).
157. Cuthbertson, D. P., et al., *Glasgow Med. J.*, **35**, 201 (1931).
158. Ellinger, A., and Matsuoka, Z.: *Z. physiol. Chem.*, **109**, 259 (1920).
159. Kotake, Y., et al., *Z. physiol. Chem.*, **195** (1931); **214** (1932)—A series of papers. cf. especially 195, 158 (1931).
160. Jackson, R. W., and Jackson, W. T.: *J. Biol. Chem.*, **96**, 697 (1932).
161. Peters, J. P., et al., *J. Clin. Investigation*, **8**, 577 (1930); **10**, 941 (1931); **11**, 97, 103, 113 (1932).
162. McCann, W. S.: *Ann. Internal Med.*, **5**, 579 (1932). Editorial, *J. Am. Med. Assoc.*, **99**, 920 (1932); Harrop, G. A.: *Diet in Disease* (1930).
163. *Ann. Rev. Biochem.*, **2**, 165 (1933).
164. Faber, H. K., and Brown, D. M.: *Proc. Soc. Exptl. Biol. Med.*, **30**, 335 (1932-33).
165. Fåhræus, A.: *Acta Med. Scand.*, **55**, 1 (1921).
166. Mitchell, H. H., and Hamilton, T. S.: *The Biochemistry of the Amino Acids* (1929); Cathcart, E. P.: *The Physiology of Protein Metabolism* (1921); Dakin, H. D.: *Oxidations and Reductions in the Animal Body* (1922); Garrod, A. E.: *Inborn Errors of Metabolism* (Oxford, 1933); Neubauer, O.: *Intermediärer Eiweiss-Stoffwechsel, Handb. norm. path. Physiol.*, **5**, 671 (1928).
167. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry* (1931).

CHAPTER XXIII

MINERAL METABOLISM

CALCIUM*

CALCIUM occupies the position of first importance with regard to the minerals. The problems involved are of great practical and theoretical interest. The large amount of calcium and the associated phosphate and carbonate in the skeleton make this organ the chief site, quantitatively, of the mineral stores. The rôle of calcium in all neuromuscular phenomena and its manifold functions in physiologic mechanisms, render it of great biological importance: Its absorption, excretion, ionization, and mode of action are fundamental physico-chemical and biochemical problems. The application of this knowledge to botany, agriculture, animal husbandry, and bacteriology, together with the other biological sciences, is becoming increasingly fruitful. Authority for many of the statements made on calcium and phosphorus will be found in the recent reviews.^{6, 33, 37}

Requirement.—Adequacy of intake has been measured by studies of balance, growth, longevity, fertility, lactation, viability and vigor of offspring, and bone and tooth structure. Sherman's original study, calculated from the food consumed, still serves as a model for this type of investigation. Similar studies, especially of children, have been reviewed.⁴⁰ The minimal and optimal intakes still remain open problems. Sherman recommends 0.75–1 Gm. per day, especially for children.

It has been demonstrated that either low calcium or low phosphorus can be a limiting factor in body growth. E. Voit, before the vitamin era, and later McCollum and associates produced, with low calcium diets, not only rickets, but lessened growth. With larger calcium intakes, larger calcium retentions occur. Better retentions also occur with the same intake where the vitamin supplements are increased.

The calcium requirement is intimately connected with the phosphorus intake. McCollum and associates, and Sherman and Pappenheimer demonstrated that, with vitamin restriction, rickets and diminution in growth could be produced with high calcium-low phosphorus diets; $\text{Ca/P} = 4$. When the latter investigators added phosphorus to change the Ca/P ratio to 2, rickets was prevented while the absolute amount of phosphorus was still smaller than in McCollum's diet. Further studies have showed that the level as well as the ratio of

* Comprehensive expositions of the subject of mineral metabolism may be found in 10, 11, 17, 22, 24, 28, 32, 39, 40.

calcium and phosphorus in the diet determines its ricketogenic properties.

In this relationship of Ca/P intakes, other metals in the same group in the periodic table may be substituted for calcium: Beryllium, lead, magnesium, strontium, and thallium. (Barium is so toxic that it cannot be administered except as insoluble salts.) Iron also acts similarly.

Calcium and phosphorus requirements in pregnancy and lactation present special problems. They have been studied in the human, dog, rat, and farm animals. During the drain of lactation, negative balances of calcium and, to a lesser extent, of phosphorus occur—regardless of the intake of minerals and vitamins.

Breast-fed babies thrive better than those fed on cow-milk modifications, although the calcium retentions of the bottle-fed babies are larger. Retentions parallel the intakes even when these vary widely. Are maximal retentions optimal retentions? What the effect in later life will be has never been shown. Do these individuals develop more arteriosclerosis, are they more subject to stone formation, or is their general well-being always improved? Sherman and Booher have stated that, with higher intakes, maturity is reached earlier and the span of life before senility increased.

Blood Serum.—Blood serum holds more calcium than can be contained in simple inorganic solution; and the various forms of calcium must be discovered before great advance can be made in understanding calcium action, either physiologically or physicochemically. Serum contains twice as much calcium as the protein-free cerebrospinal fluid, which indicates that a considerable portion of calcium is bound to protein.* The nonprotein calcium is inverse in amount to the phosphate. But this does not describe the complete picture, for in the blood of children the protein and calcium are approximately the same as in adults, but the phosphate may be doubled. Carbohydrate ingestion lowers phosphorus, but does not usually raise calcium, and both calcium and phosphorus may rise or fall together, irrespective of the protein change. That citrate-like calcium compounds occur has been suggested and denied.

Serum calcium may be divided into diffusible and nondiffusible portions, the former carrying the ionic and the latter the protein-bound. This crude separation has not proved adequate; for calcium injections, parathormone and vitamin D raise both fractions. There is no reliable method for measuring ionized calcium. Klinker has suggested that part of the calcium may be in negatively charged combination, because by electrophoresis experiments it goes to the anode and also is adsorbed on positively charged adsorbents such as barium sulfate. Benjamin and Hess⁴ have recently divided the calcium into four portions: In addition to the ionic and protein-bound calcium, there are two forms of adsorbable, one diffusible and the other non-

* But consider amniotic fluid, which has a high calcium value, but no protein.

diffusible. The adsorbable fraction (not ionic) is diminished in rickets. Adsorption also removes a part from inorganic solutions. The laying hen presents a particular problem, for then the blood calcium is about double that normally found. Here too Benjamin has found that the adsorbable fraction is the chief variable. Thus a new approach has been developed.

Parathyroid and Vitamin D.—Whereas a few years ago we were unaware of the regulators of calcium metabolism, we now have an *embarrass de richesse* with both vitamin D and parathormone. The present relation of these two potent factors is in an interesting situation.³⁷ No one knows how either acts, or whether they produce their effect by interaction. Neither added *in vitro* to inorganic solutions will cause precipitation of bone salts in cartilage. *In vivo*, either in excess will raise serum calcium and cause decalcification. Large doses of vitamin D will prevent and cure tetany in parathyroidectomized animals. Further, the action is related to the calcium and phosphorus intakes. The trend of opinion is to regard vitamin D as favorably affecting the "net absorption" of calcium from the gut.¹⁵ But parathormone and vitamin D act in the absence of intestines, or in fasting animals. Vitamin D in optimal doses increases calcium retention; parathormone causes negative calcium balances and breaks down bone material. Parathormone causes a preliminary increase in magnesium and decrease in the phosphate of the blood serum followed by a rise of calcium. The method of absorption and mode of action of irradiated ergosterol remain undetermined.

Thyroid.—Another regulator of calcium metabolism is found in the active principle of thyroid, which in therapeutic doses causes an increased excretion of calcium in urine and feces without raising low serum calcium. This, too, withdraws calcium from the bones.¹

Bone.—That the salts in the bone matrix are not simply $\text{Ca}_3(\text{PO}_4)_2$ and CaCO_3 , modern investigators are agreed. x -Ray studies and indices of refraction strongly indicate an "apatite" structure. For physicochemical studies of equilibration such knowledge is prerequisite. The trabeculae are the mobile sites of calcium deposition.³

Calcification.—The factors in normal calcification involve at least the following: (a) Intakes of calcium and phosphorus; (b) ratio of Ca/P; (c) effect of heavy metals; (d) acid-base; (e) phosphatase; (f) vitamin C; (g) vitamin D; (h) thyroid; (i) parathyroid; (j) other glands. Many of these factors are interdependent. In addition, there are the special problems of calcification *in vitro*²¹ pathologic calcification² and that due to excess irradiated ergosterol, healing fractures and rickets.

New theories of calcification are springing up regarding the formation of parathormone compounds, the effect of the pituitary and the effect of lymph gland extracts. Thus, adequate physicochemical explanations of the ionization of Ca^{++} , PO_4^{---} , HCO_3^- , H^+ , and the solubility product of insoluble calcium salts still remain in prospect.

Teeth.—Faulty calcification of teeth, and especially the cause and prevention of dental caries, have been the subject of many recent investigations. Mrs. Mellanby²⁵ lays the blame principally on vitamin D. Vitamin A, vitamin C, Ca/P, heredity, candy, soft food, particle size, general health, general diet, and many other causes have been indicted. Where there are so many explanations, it is obvious that the problem is still unsolved.

Diseases of Calcium and Phosphorus.—The diseases of calcium metabolism and bone have been recently reviewed.¹⁹ The most important of these theoretically, experimentally and practically is rickets. The most careful and exhaustive review of experimental rickets is that of Goldblatt;¹² of clinical rickets, those of Hess¹⁶ and György.¹⁴

Tetany is a condition of hyperirritability of the neuromuscular system, probably in the nerves. Infantile tetany is closely associated with healing rickets. It has been described as due to excess phosphate retention in healing rickets. Phosphate by mouth or injection, fasting of ricketic animals (with the production of PO_4 from the tissue breakdown), or healing of mild rickets in rats all produce this condition. In addition to such tetany, the symptom complex follows parathyroidectomy, hyperventilation, alkalosis brought on by vomiting, oxalate administration, citrate injection, high-phosphorus or low-calcium intake, and withdrawal of parathyroid, or the operation in Recklinghausen's disease.

MAGNESIUM

A new form of tetany which follows low magnesium intake has been described by McCollum and associates. Thus magnesium becomes an element essential to life; for when the intake is as low as 1.8 p.p.m. tetany and death follow. The serum calcium remains normal; therefore it is necessary to abandon theories of tetany dealing only with calcium ionization, and to return to the older concept of irritability as the sum total of the mineral environment.*

SODIUM

The sodium of the body, together with the chlorine, serves largely to maintain the osmotic and base equilibrium of the body fluids. The relation to water metabolism is intimate; the main interest centers about the maintenance of blood volume, the storage of fluids in the tissues, as in edema and dehydration, and the laws governing excretion by the kidneys and skin.

Authority for the following statements will be found in the recent review.³⁴ There are other résumés.^{22, 26, 28}

Blood.—The sodium of the blood serum comprises about 93 per cent of the total bases. The relation of the cerebrospinal fluid sodium and chlorine to serum has been reported to be not inconsistent with the Donnan equilibrium.

* Reviews of the recent work on magnesium have been made.^{22, 28, 33}

Metabolism.—The sodium salts are easily absorbed, circulate readily, and are excreted principally by the kidneys—normally 90 per cent. Rats on low salt diets, sufficient to maintain life but not growth, excrete only traces of base in the urine. The dictum of Bunge that, in normal men, large intakes of sodium cause excretion of potassium, has recently received both confirmation and denial. Acid or acid-forming salts lead to an excretion of base (and dehydration). Sodium chloride may lead to production of acidosis in infants. It may be retained without water in infancy, in epilepsy and in pneumonia. Addition of sodium chloride to replace that lost in sweat leads to relief of fatigue and dehydration.

Edema has been produced in experimental animals by low protein diets and adequate salt intake, or by plasmaphoresis. The effect depends more upon the sodium than upon the chlorine. When sodium chloride is excreted by the kidney water is also removed and diuresis thus produced. The action of the kidney must be considered from the viewpoint of both the sodium and the chlorine to be excreted. Studies have shown that diuresis in epilepsy and in nephritic edema lead to loss of sodium chloride; even with pituitary antidiuresis sodium chloride is excreted in excess. Parathyroid extract also produces diuresis.

An outstanding recent contribution is the discovery of the control of sodium metabolism by the adrenal cortical hormone.

POTASSIUM*

The uneven distribution of potassium and sodium, the latter predominantly in the body fluids and the former in the cells, remains an unsolved mystery. The nature of the combination and the function of potassium in muscle are not understood. The finding of Neuschloss of "bound potassium" in rabbits' muscle has not been confirmed. The potassium is said to be present in indiffusible form, and to be liberated during contraction. The potassium content of red and white muscles and of heart and stomach varies over 100 per cent even in different pieces from the same muscle. Comparisons of potassium in normal and pathologic heart muscle have yielded conflicting data.

Blood.—The distribution of potassium between cells and serum differs according to species. The potassium content of about 20 mg. per cent in human serum shows slight variations under any conditions. A rise has been found in adrenal insufficiency. It has been found that the more rapidly plasma is separated from cells, the less potassium it contains. The thesis that serum potassium is in part colloidal was deduced from ultrafiltration experiments. Serum contains more potassium than cerebrospinal fluid or pleural effusions.

Much interest has been shown in the ratio of potassium to calcium, because of their well-known reciprocal relationship to irritability, and of potassium to sodium in adrenal insufficiency and shock.

* There are several comprehensive and recent reviews which may be consulted for details of the following discussion. ^{22 26 28 34}

Metabolism.—Long-continued ingestion of small amounts of potassium is said to increase the content of milk, kidneys and heart. Potassium is excreted preponderantly in the urine. In nephritis it is eliminated with difficulty. Potassium salts have long enjoyed an important place as diuretics. In mineral balances the potassium in sweat must be taken into account.

CHLORINE*

That the skin and subcutaneous tissue may serve as a depôt for chlorine is well established. This chlorine is not replaceable by administered iodine, even under condition of need for chlorine, as in the edema caused by superficial burns.

Blood and Cerebrospinal Fluid.—In addition to its importance in osmotic relationships, chlorine can pass across the "cell membrane," whereas sodium and potassium cannot, and hence functions in acid-base and Donnan equilibria. It forms about two-thirds of the acids of the blood serum. Taken together with sodium bicarbonate, with which it varies inversely, the stability of the blood acids is maintained.

The red cells contain only about half as much chlorine as plasma. The distribution of chlorine (and bromine) follows the Donnan membrane equilibrium *in vitro* but not *in vivo*, or in various diseases. The diminution of chlorine has been reported in pneumonia, in tuberculosis, in malaria and in adrenal insufficiency; and an increase in scurvy. The effect of sodium chloride in reducing blood sugar in diabetes, reported by Peters, has been confirmed.†

The chlorine in cerebrospinal fluid or in ultrafiltrate exceeds that in serum. Similar relations in nephritis and NH_4Cl acidosis have been found.

Gastric Secretion.—Gastric acidity is not influenced by sodium chloride intake but is affected by the composition of the blood. Under histamine stimulation, the secretion of gastric juice has been shown to be of constant chlorine content and acidity. The variations usually found are attributed to mucus. With vomiting, as in high intestinal obstruction or water intoxication, chlorine is lost from the body; the blood and tissue chlorides are diminished.

Metabolism.—About 10–15 Gm. of sodium chloride constitute an average daily consumption. Low salt intakes lead to a practical cessation of excretion; such diets have been recommended in tuberculosis. High salt diets cause increased water consumption and even in normals may lead to retention or edema. Increased salts in drinking water lead to increased fluid intakes, up to a concentration of

* Little has been written recently that changes the information summarized by Peters and Van Slyke,²⁸ Chapter XIX, and Klinker,²³ Chapter IX. The nature of chlorine compounds is under examination and the proof of organic combinations would be welcome, but convincing evidence is not at hand²³ (footnote, p. 1019).

† An excellent account of chlorides in the blood in nephritis has been given by Peters and Van Slyke,²⁸ Chapter XIX.

1.3–1.7 per cent; animals die of thirst or dehydration rather than drink such a solution.

Chlorine is easily absorbed into the blood and is excreted normally almost wholly in the urine. The laws governing excretion are not as simple as Ambard once supposed, for Peters and others have shown that chlorine may be excreted when the serum chloride is below the "threshold" value. The relations of the anterior and posterior hypophysis to diuresis are especially important. The amounts of chlorine in feces and sweat show variation, but are usually small; but in profuse sweating the chlorine thus lost may exceed that in the urine.

IRON

The metabolism of iron is concerned not only with hemoglobin, and hence the oxygen- and carbon dioxide-carrying, and acid-base properties of the blood²⁸ (Chapters XII and XVII), but also with oxidation in every cell. Bunge and Sherman have constantly maintained that it constitutes a major problem in nutrition.*

Occurrence.—Macallum, by classical microchemical studies in 1894, demonstrated that the cell iron is located in the chromatin. The iron in blood-free tissues could not be depleted by diet or anemia. The liver is the main organ of storage, and the spleen and kidneys are secondary. Iron may be found in calcified aortas and in gallstones. The iron content of the newborn of various species and its increase with age on various diets have been determined. The content of the placenta in relation to both fetus and mother has been studied, and found to parallel that of the fetus.

Metabolism.—Iron occurring in foodstuffs is readily available. Inorganic ionizable forms of iron seem to be better utilized than citrates, lactates and ferricyanides, and these better than hemoglobin. "Active" iron has different properties from ordinary iron. It is stated that a reduction to Fe^{++} precedes assimilation. Practically no iron is normally excreted in the urine; that absorbed is later excreted into the gut.

The iron requirement for the adult may be as low as 1 mg. per day, but 10–15 mg. are desirable. The demands of pregnancy, infancy, prematurity, and childhood have been investigated.⁴⁰ In the light of the above, the danger of diets being too low in iron has been exaggerated, except in exclusive milk diets. Human milk normally contains about 1.5–2 mg. per liter, and cow milk 0.5 mg. Iron intake influences the iron content neither of milk nor of eggs.

Blood.—The iron released by normal destruction of blood cells is not excreted but reutilized; that freed by toxic destruction may be held tenaciously in reserve in the liver. How this intermediary met-

* There are a number of recent reviews for both humans and animals,⁴⁰ including the relation of the spleen to iron metabolism. The voluminous literature on the chemistry and physiology of hemoglobin forms a special topic and is not here included. (See Chapters XV to XIX.)

abolism is accomplished is unknown: Little iron is excreted in the bile, the leukocytes seem too few to account for the transport, and the hemoglobin has not been shown to take part. Therefore some attention has been given to the serum. The iron content of serum has been described as ferric; and ultrafiltrable. The importance of serum iron is as yet unknown.

Nutritional Anemia.—Of the many clinical forms of anemia, only one can be mentioned here. That nutritional anemia is produced in both infants and experimental animals by an exclusive milk diet is now well known. It was tacitly assumed that it was caused by iron deficiency until, by their dramatic statements in 1928, Hart and Steenbock focused attention on the importance of copper in nutritional anemia.^{29, 35, 40} The Wisconsin investigators found that iron plus 0.01–0.02 mg. of copper per rat per day would cure this condition. These claims have been amply confirmed.

Considerable controversy has arisen on two points in nutritional anemia of rats: (1) Whether other traces, especially manganese, have supplemental action; (2) whether iron alone will cure anemia. Myers and Beard have answered both questions affirmatively, but now remain practically alone in these claims. Infants can be cured of anemia by pure iron. A report has been made of cure by intraperitoneal injection of iron alone in both infants and rats.

The copper in the best milk provides approximately half the amount required. If an adequate copper-free, non-milk diet could be devised, the problem would be advanced.

The mechanism of this hematopoietic action has been investigated. Iron may be stored in the liver without increase in hemoglobin; when copper is given the stored iron is utilized.

IODINE

The account of the relation of iodine to the thyroid gland of man and animals constitutes one of the brilliant chapters of modern physiology.*

Iodine is found in the thyroglobulin of the thyroid gland, from which the active principle, thyroxine, was isolated and crystallized by Kendall in 1915. Harington and Barger proved its structure by synthesis. A milligram of this compound exerts a profound effect upon basal metabolism. Diiodotyrosine, which is inert, has also been separated from thyroglobulin. Of the total thyroid iodine, only 20–25 per cent is thyroxine; but the activity is greater than that accounted for by the thyroxine content alone. The thyroid of man contains about 15 mg. of iodine, which constitutes over half that in the body.† Next to the thyroid in quantity of iodine are found, in diminishing order, ovary, adrenal, and thymus.

* Recent reviews have been given.^{28, 27, 33, 40}

† A review of the iodine content of the thyroid in pathologic conditions will be found in^{13, 20}.

Blood.—The blood iodine content of about 10–12 γ per 100 cc. is divided into organic and inorganic, alcohol-soluble and insoluble. The blood iodine varies with the season. It is increased in exophthalmic goiter and toxic adenomata, and diminished in simple goiter and myxedema. Injected or ingested iodine is partly retained by the thyroid. The relation I/Br is constant, and also I/Cl in skin. Adrenalin causes a transitory rise in blood iodine.

The iodine content of the cerebrospinal fluid, normally 7 γ , is always less than that of the blood.

Intake.—The intake of iodine depends upon the location of the food supply, water supply and air—which are all dependent upon the soil. McClendon has made an extensive study of the geographical distribution of iodine. At least 50 γ —1 mg. should be ingested daily—probably more in childhood and during puberty, gestation, lactation and infections. The question of the best source of iodine is tinged with commercial exploitation. The danger of iodism from overdosage in humans and animals seems remote under the present circumstances.

Goiter.—Restriction of iodine intake results in deficiency of the thyroid gland; and abortion and fetal death in cattle, and goiter in humans commonly follow. The relation of iodine deficiency to simple goiter is beyond dispute, due to the classic investigations of Marine²³ and the prophylactic experiment on the school children of Akron by Marine and Kimball. However, lack of iodine is not the only cause of thyroid enlargement. In areas where iodine is administered there is a residual of goiter. Cyanides and cabbage (fat) have been found to be goitrogenic. The ricketogenic diet of Steenbock and Black, with or without vitamin D, caused thyroid hyperplasia in rats,³⁶ and has therefore been used as a diet for standard goiter production.

More extreme thyroid deficiency results in hypothyroidism, as in myxedema and cretinism, with lowered basal metabolism, diminished growth and intelligence, coarse skin and hair. Hyperthyroidism is recognized by increased basal metabolism, rapid heart rate, exophthalmus, tremor, sweating and loss of weight.

Metabolism.—Free iodine or iodides are rapidly absorbed, even from mucous surfaces or skin, and circulate in all the body fluids (even in myxedema). They are readily excreted, on liberal intakes chiefly in the urine; but the feces, sweat, tears, saliva, and bile contain small amounts.

Ovarian hormone increases iodine excretion, either through direct action on the thyroid or indirectly through the anterior pituitary.³⁰ *

In lactating animals, the amount of iodine in the milk depends upon the intake.^{5, 8} The mother transmits iodine to the fetus in proportion to her supply.^{9, 18} This is also true of hens, which, after administration of iodine or thyroxine, laid "iodized" eggs.

* The effect of iodine and thyroxine on the thyroid, hypophysis, and nervous system has been reviewed.⁷

TRACES

In nutrition studies "the importance of little things" is assuming major proportions.* Many substances occur in the body in such minute amounts that their presence may be due to accidental contamination. A study of them belongs specifically to pharmacology and toxicology. Others, such as aluminum, arsenic, bromine, cobalt and nickel, fluorine, manganese, silicon and zinc, have received considerable attention in the last few years.

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REFERENCES

1. Aub, J. C., Albright, F., Bauer, W., and Rossmeis, F.: *J. Clin. Invest.*, **11**, 211 (1932).
2. Barr, D. P.: *Physiol. Rev.*, **12**, 593 (1932).
3. Bauer, W., Aub, J. C., and Albright, F.: *J. Exptl. Med.*, **49**, 145 (1929).
4. Benjamin, H. R., and Hess, A. F.: *J. Biol. Chem.*, **100**, 27 (1933).
5. Brouwer, E.: *Arch. néerland. physiol.*, **16**, 299 (1931).
6. Cantarow, A.: *Calcium Metabolism and Calcium Therapy* (1933).
7. Closs, K., Loeb, L., and MacKay, E. M.: *J. Biol. Chem.*, **96**, 585 (1932).
8. Courth, H.: *Biochem. Z.*, **238**, 162 (1931).
9. Culbertson, C. C., and Thomas, B. H.: *Iowa Agr. Expt. Sta. Ann. Rept.*, **30** (1931).
10. Czerny, A., and Kellar, A.: *Des Kindes-Ernährung, Ernährungsstörungen und Ernährungstherapie* (1923-1928).
11. Forbes, E. B., and Keith, M. H.: *Agr. Expt. Sta. (Ohio) Tech. Series Bull.*, **5**, 1 (1914).
12. Goldblatt, H.: *Ergebnisse allgem. Path. u. path. Anat. Menschen u. Tiere*, **25**, 58 (1931).
13. Gutman, A. B., Benedict, E. M., Baxter, B., and Palmer, W. W.: *J. Biol. Chem.*, **97**, 303 (1932).
14. György, P.: *Handb. norm. path. Physiol.*, **16** (Part 2), 1555 (1931).
15. Harris, L. J.: *Annual Rev. Biochem.*, **1**, 337 (1932); **2**, 253 (1933).
16. Hess, A. F.: *Rickets Including Osteomalacia and Tetany* (1929).
17. Heubner, W.: *Handb. norm. path. Physiol.*, **16** (Part 2), 1419 (1931).
18. Hudson, G. E.: *J. Am. Med. Assoc.*, **97**, 1513 (1931).
19. Hunter, D.: *Quart. J. Med.*, **24**, 393 (1931).
20. Jordi, A.: *Arch. Internal Med.*, **49**, 541 (1932).
21. Kay, H. D.: *Physiol. Rev.*, **12**, 384 (1932).
22. Klinke, K.: *Der Mineralstoffwechsel. Physiologie und Pathologie* (1931).
23. Marine, D.: *Medicine*, **6**, 127 (1927).
24. Mattill, H. A., and Mattill, H. I.: In Barker, L. F.: *Endocrinology and Metabolism* (1922).
25. Mellanby, M.: *Physiol. Rev.*, **8**, 545 (1928).
26. Meyer-Bisch, R.: *Handb. norm. path. Physiol.*, **16** (Part 2), 1517 (1931).
27. Orr, J. B., and Leitch, I.: *Medical Research Council (London) Special Report Series*, No. 123 (1929).
28. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry* (1931).
29. Rose, M. S.: *J. Nutrition*, **1**, 541 (1929).
30. Scheringer, W.: *Arch. Gynäkol.*, **146**, 248 (1931).
31. Sherman, H. C.: *Chemistry of Food and Nutrition* (1932).
32. Shohl, A. T.: *Physiol. Rev.*, **3**, 509 (1923).
33. Shohl, A. T.: *Annual Rev. Biochem.*, **2**, 207 (1933).
34. Shohl, A. T.: *Annual Rev. Biochem.*, **3**, 209 (1934).
35. Smith, A. H.: *Annual Rev. Biochem.*, **1**, 319 (1932); **2**, 299 (1933).
36. Thompson, J.: *J. Nutrition*, **5**, 359 (1932).
37. Thomson, D. L., and Collip, J. B.: *Physiol. Rev.*, **12**, 309 (1932).
38. Von Fellenberg, T.: *Ergebnisse Physiol.*, **25**, 176 (1926).
39. Wendt, G. v.: In Oppenheimer, C.: *Handb. Biochem.*, **8**, 183 (1925).
40. White House Conference on Child Health and Protection. Growth and Development of the Child. Part III. Nutrition (1932).

* Reviews have been made by the White House Conference,⁴⁰ p. 282, Klinke,²² Chapter XIV, and Rose.²⁹

CHAP₂ XXIV

BONE AND TEETH

BONE and teeth, classified as connective tissue, are unique in being composed of organic and inorganic matter intimately mixed or probably combined. Since 1914 the development and the abnormal conditions of these tissues have been the subject of many investigations; but since the problem is a difficult one there is much yet to be done before a satisfactory solution is obtained.

To present this section in as simple a manner as possible, it is proposed to divide the subject matter into three parts: (1) The calcified tissues of invertebrates; (2) the normal calcified tissues of vertebrates; (3) the abnormal calcified tissues of vertebrates.

INVERTEBRATES

These are usually provided with an exoskeleton which is epiblastic in origin, and less frequently, with a simple skeleton of similar substance. Many of these protective coverings consist of chitin¹ either alone or in association with siliceous or calcareous material; but in certain phyla of invertebrates, scleroproteins and chitinoids are found which are in some cases impregnated with calcium salts. In the latter classes, gorgonin of corals, spongin of sponges,^{2, 3} onuphin of tubicola, conchiolin of lamellibranchii are examples.

The table (p. 656) gives the results of the analysis of these scleroproteins (or albuminoids) and chitinoids. Other proteins are included for purposes of convenience, as they will be discussed later.

None of the scleroproteins or chitinoids found in the invertebrate kingdom resemble chitin, for the latter gives, on acid hydrolysis, glucosamine and acetic acid only. Conchiolin only is in any way like chitin. It contains a larger amount of nitrogen; and when boiled with dilute sulfuric acid gives glycine, leucine and tyrosine in abundance, but no sugar. Spongin is unlike the scleroproteins found in mammals in that it does not give gelatin when boiled with water. It is readily soluble in caustic alkalis and dissolves with difficulty in concentrated mineral acids. The chief products obtained on hydrolysis are glutamic acid and glycine.

Chitin¹ is very closely related to the mucins.⁹ The former supposed to be polymerized monacetyl glucosamine, yields on hydrolysis glucosamine and acetic acid. The latter belongs to the class named mucoproteins which are conjugated proteins, and from the work so far carried out it has been shown that they may be split up into mucoitin or chondroitin sulfuric acid and a protein. The great number and variety of the mucoproteins depend upon the protein part of the complex molecule. The mucoitin sulfuric acid, on further hydrolysis, yields

| | <i>Spongin</i> ^{2, 3} | <i>Gelatin</i> ⁴ | <i>Ichthylepidin</i> <i>from fish</i> <i>scales</i> ⁵ | <i>Elastin</i> ^{6, 7, 8} |
|--------------------------|--------------------------------|-----------------------------|--|-----------------------------------|
| Glycine..... | 13.9 ² | 25.5 | 5.7 | 25.8 ⁶ |
| Alanine..... | | 8.7 | 3.1 | 6.6 ⁶ |
| Valin..... | | 0 | | 1.0 ⁶ |
| Leucine..... | 7.5 ² | 7.1 | 15.0 | 21.4 ⁶ |
| Isoleucine..... | | | | |
| Phenylalanine..... | | 1.4 | | 3.9 ⁶ |
| Tyrosine..... | 0 ² | 0 | 1.0 | 0.4 ⁷ |
| Serine..... | | 0.4 | | |
| Cystine..... | | 0 | | |
| Proline..... | 6.3 ² | 9.5 | 6.7 | 1.7 ⁶ |
| Hydroxyproline..... | | 14.1 | | |
| Aspartic acid..... | 4.7 ² | 3.4 | 1.2 | + |
| Glutamic acid..... | 18.1 ² | 5.8 | 9.2 | 0.8 ⁶ |
| Hydroxyglutamic acid.... | | 0 | | |
| Tryptophan..... | | 0 | | |
| Arginine..... | 5.5 ² | 8.2 | | 0.3 ⁷ |
| Lysine..... | 3.5 ² | 5.9 | | + |
| Histidine..... | 0 ² | 0.9 | | + |
| Ammonia..... | | 0.4 | | |
| Total..... | 59.5 | 91.3 | 42.0 | 61.9 ⁷ |

four components in equimolecular proportions; *viz.*, sulfuric acid, acetic acid, glycaronic acid and chitosamine (2-glucosamine).⁹

The prosthetic groups so far isolated from glucoproteins are mucoitin sulfuric acid and chondroitin sulfuric acid. The latter is present in cartilage, thus showing a close relationship between the skeletons of invertebrates and vertebrates. The only difference in the structure of chondroitin and mucoitin sulfuric acid is in the nature of the hexosamine, the former being 2-galactosamine (chondrosamine) and the latter 2-glucosamine (chitosamine).

NORMAL CALCIFIED TISSUES OF THE VERTEBRATES

The distribution of chitin is limited to those animals belonging to the invertebrate kingdom. In the higher divisions of the animal world,

chitin is replaced by cartilage which is either partly or wholly ossified to form bone or teeth. In the elasmobranchii, the endoskeleton is practically all cartilage, but the exoskeleton consists of dermal ossicles which are similar to mammalian teeth, consisting of an outer layer of enamel enclosing dentine and pulp cavity. These serve as teeth in the region of the jaws. In the teleostomi the skeleton is more or less ossified. It is said that the bones of fishes contain a greater proportion of water, organic matter and soluble salts than the bones of other vertebrates.

Morgulis,¹⁰ after studying the compositions of the bone ash from a variety of fishes and mammals, reported that the proportion of calcium carbonate in the former is about half that in the latter. A protein named "ichthylepidin" has been found in the scales of teleostomi. The amount is 24 per cent; the remainder of the organic matter is collagen. This protein is, apparently, absent in the elasmobranchii.

The analysis of ichthylepidin⁵ is given on page 656. From these results and from the work of Green and Tower¹¹ it may be concluded that ichthylepidin is closely related to elastin and keratin. Doubtless, it is epiblastic in origin and is therefore more closely related to the keratins.

The Bones of Amphibians, Reptiles and Birds.—The bones of amphibians, like those of fishes, are found to contain a high proportion of organic matter as well as sodium sulfate. There is little known concerning the calcified tissues found in reptiles. The endoskeletons of birds are interesting as they contain no marrow and are composed of a greater proportion of cancellous tissue and a higher percentage of water than those of mammals. Silicic acid is usually found in the skeletons of granivorous birds.

Mammalian Bones and Teeth.—This section dealing with the growth of bones and teeth in mammals is of great importance, as it includes the metabolic changes taking place in these tissues in human beings. The problem resolves itself into the chemistry of cartilage, collagen, mucoids and the inorganic substances associated with these compounds to form bone and teeth.

Mammalian Bones.—Bones are the supporting tissues of the body and, in order to get rigidity as well as hardness, nature has provided that a living tissue, consisting of both organic and inorganic matter, has been developed, and designed in such a way as to give great strength. It has been found by calcination of bone that the resulting ash contains principally calcium, phosphate, carbonate and small amounts of magnesium, chlorine and fluorine. The organic matrix, freed from the mineral matter by treating the tissue at ordinary temperature with frequent changes of dilute hydrochloric acid over a period of several days, is composed of a protein (ossein), a mucoid (osseomucoid) and a keratin (osseo-albuminoid). These inorganic and organic substances present in mammalian bones will be discussed in the following paragraphs.

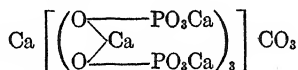
Bone Earths of Mammalian Bones.—Originally, the inorganic constituents were considered to consist of a calcium phosphate, magnesium phosphate and calcium carbonate with traces of chloride and fluoride. The following is a typical analysis of the mineral matter of the bones of vertebrates:

| | Per cent. |
|------------------------------------|-----------|
| $\text{Ca}_3(\text{PO}_4)_2$ | 85.0 |
| $\text{Mg}_3(\text{PO}_4)_2$ | 1.5 |
| CaF_2 | 0.3 |
| CaCl_2 | 0.2 |
| CaCO_3 | 10.0 |
| Alkaline salts..... | 2.0 |

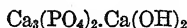
The mineral constituents of bone earths exist in rather constant proportions and are nearly the same in different animals. It was on this account that Hoppe-Seyler suggested that the ratio $\text{Ca}:\text{PO}_4$ was 10:6 as in apatite; i. e., about 3 molecules of $\text{Ca}_3(\text{PO}_4)_2$ to 1 molecule of CaCO_3 .

The nature of the so-called "bone earths" has been attacked by using both chemical and physical methods, and, from the results so far published, it may be concluded that no satisfactory solution has yet been advanced.*

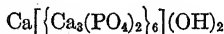
Chemical Methods.—By using chemical methods Gassmann¹³ suggested the following composition:



This has been criticized by Klement¹⁴ who maintains that $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ is first laid down in the organic matrix, which is then subsequently hydrolyzed to basic calcium phosphate:



At the same time a variable amount of calcium carbonate and alkali hydrogen carbonate is also deposited. Shear and Kramer,¹⁵ by studying the Ca/P ratio in normal adult bone, conclude that $\text{Ca}_3(\text{PO}_4)_2$ is the chief constituent probably combined in some complex manner with CaCO_3 ; but that CaHPO_4 may also be present. The presence of a basic secondary phosphate has been suggested. Another formula brought forward is



Physical Methods.—The determination of refractive indices and x-ray spectra analysis are the physical methods which have been used in these investigations. Taylor and Sheard¹⁶ adopted the former method for the examination of various samples of dried bone and compared the result obtained with those of minerals of known composition; dahlite, $\text{CaCO}_3 \cdot 3\text{Ca}_3(\text{PO}_4)_2$, was selected. Rosebury, Hastings

* See the review by Kay.¹²

and Morse¹⁷ studied the problem by the x-ray spectra method. They conclude that (a) bone has a crystalline structure which is fundamentally that of other members of the apatite series; (b) the formula $\text{CaCO}_3 \cdot n[\text{Ca}_3(\text{PO}_4)_2]$ represents the calcium ratio in bone and enamel where n is not less than 2 nor greater than 3; (c) there is no evidence of either free CaHPO_4 or free CaCO_3 in bone.

Variation of Mineral Content of Bones with Age.—The effect of age on bones may be generalized as follows: The percentage of water decreases and the ash content increases with age; but the variations in the percentage of organic matter are not strongly marked.

Other Elements Present in Bone.—Other elements have been detected in bone and teeth; *viz.*, fluorine, strontium and lithium. The last two have been detected in human calcified tissues. Strontium cannot replace calcium physiologically in bone formation. The amount of fluorine in bones and teeth is said to vary in different species. Some believe that fluorine is not present in these tissues in young animals but is found in increasing quantities as the animal grows.

Fluorides, it is claimed, cause the inhibition of normal growth and marked resorption of normal bone. Results have been published showing the difference in the fluorine content of the bones and teeth of land and sea animals. The former contain 0.03–0.065 per cent, whereas the latter contain more than 0.57 to 1.62 per cent of fluorine. Fossilized bones contain as much as 2.73 per cent of this element.

Organic Matrix of Mammalian Bones.—As stated above, the organic matrix of bone is composed of a mixture of proteins; *i. e.*, a scleroprotein, a mucoprotein and a keratin (?). The scleroprotein is *ossein*, which is very similar to the collagen of tendons and skin; the keratin is a very tough elastic and fibrous substance similar to elastin found in connective tissue and tendons and which, for convenience, will be named *osseo-albuminoid*. The mucoprotein named *osseomucoid* belongs to the class of conjugated proteins (glucoproteins) and resembles *tendomucoid* found in tendons.

These substances may be separated by the method given by Knaggs and Schryver¹⁸ and Knaggs¹⁹ which consists in the removal of the *osseomucoid* by prolonged treatment with dilute caustic soda.

To free the *ossein* from the *osseo-albuminoid*, the latter may be removed by tryptic digestion, after treatment with alkali.²⁰ The *osseo-albuminoid* may be obtained as an insoluble residue by extracting the casein with many changes of hot water.

Osseomucoid.—This substance was studied by Hawk and Gies²¹ who showed that on boiling with dilute hydrochloric acid it gave a reducing substance and sulfuric acid. They also determined its elementary composition; but it was not until Levene and his coworkers investigated the problem that a structural formula was assigned to the prosthetic group. Levene⁹ did not actually investigate *osseomucoid* but he assumed that from its great similarity to *tendomucoid* it should have a similar structure. As stated above, *osseomucoid* on careful

hydrolysis, should give a protein and chondroitin sulfuric acid, the composition of which has already been given.⁹

Ossein.—It is an accepted fact that cartilage is converted into ossein during the process of ossification; in other words, cartilage is the precursor of ossein. The cartilages from different tissues were examined by Carl Morner, and he was the first to isolate from them pure chondroitin sulfuric acid. Schmiedeberg assigned the formula $C_{18}H_{27}NSO_{17}$ to chondroitin sulfuric acid and obtained as cleavage products (using dilute mineral acids) sulfuric acid, acetic acid and chondrosin ($C_{12}H_{21}NO_{11}$).

It is now known that cartilage is a complex substance consisting of a protein and chondroitin sulfuric acid. The latter was shown to be composed of sulfuric acid, acetic acid, glycuronic acid and chondrosamine in equimolecular proportions. This structure shows the close relationship between chitin of the invertebrates and cartilage of the vertebrates.

During ossification of the tissue, the cartilage, through a process of inter- and intramolecular changes and amination due to the activity of the osteoblasts, is apparently converted into ossein and osseomucoid. At the same time the inorganic salts are laid down.

Much research has been carried out in the past on both the physical and chemical properties of collagen, and, more particularly on its derivative, gelatin, with a view to solving their molecular structure. Gelatin has been chosen for investigation more frequently, as it is a dispersoid colloid; and consequently, more physical data can be obtained from it than from its precursor, which is insoluble in water at ordinary temperatures.

Gies and his coworkers²² contributed to the earlier work on collagen and gelatin.

The percentages of glycine, proline and oxyproline isolated from gelatin are very large compared with the amount obtained from other proteins. Further, tyrosine, tryptophan and phenylalanine are absent. The large amount of glycine may be derived during ossification from the acetyl groups present in cartilage. It is difficult to explain the presence of the proline and oxyproline, but it may be suggested that these compounds are probably formed from open-chain compounds by the drastic method of acid hydrolysis, which is generally used for splitting proteins into their simpler constituents.¹⁸

Pure gelatin has a very low percentage of free amino nitrogen, which probably explains the inactivity of ossein and other collagens. Further, on hydrolysis, both gelatin and its precursor show a low amide nitrogen value. Both gelatin and its precursor are very sensitive to the action of acids, alkalis and enzymes, in that the distribution of nitrogen and also the physical properties, such as swelling, emulsifying power, etc., are considerably altered. This may be due to intramolecular rearrangements taking place in these complex molecules when treated as above.

It has been shown that gelatin, even when highly purified, is a very labile compound and cannot be regarded as a chemical entity.¹⁸ Further, gelatins extracted from their precursors that are taken from different tissues, and also from various vertebrate animals, differ in both physical and chemical properties.

Gerngross and his coworkers^{30, 31} have studied the structure of collagen and gelatin by x-ray analysis, and they show that these compounds have a high degree of orientation; the structure of collagen is very similar to that of gelatin. Gelatin exhibits more of the so-called "amorphous structure" and less of the crystalline.³²

Osseo-albuminoid.—This protein is supposed to be derived from the haversian canals. The work of Hawk and Gies³³ showed that it is very similar to chondro-albuminoid and closely related to elastin. *Elastin* is a very resistant substance. Boiling water has no effect and dilute acids have very little; but it is slowly dissolved by alkalis. Marriott³⁴ showed that both pepsin and trypsin are able to break down elastic fibers; and the ease with which these are digested depends on their source. The diamino acids are practically absent in the products obtained after the hydrolysis of these compounds. The amounts of glycine and alanine are abnormally high compared with other proteins.

Mammalian Teeth.—The following substances are present in all teeth: Enamel; dentine; cementum; and the pulp of formative tissue of the denture.

With regard to their origin and structure, hair and teeth may be considered to be very similar for the following reasons: Both hair and teeth are composed of an epithelial structure resting on a papilla of connective tissue. The former is horny (keratinous), whereas in the latter the cells are more or less calcified. The tissues from the teeth of different animals differ in structure when studied microscopically, but from a biochemical standpoint certain characteristics are very persistent and characteristic of each.³⁵

Enamel.—This is the hardest tissue found in the animal body, and its presence in teeth gives them their great resistance to wear. It contains only 5 per cent water and 3.5 per cent organic matter, the remainder being inorganic salts. The following is a typical analysis of the enamel of teeth:³⁶

| | Per cent. |
|-------------------------------------|-----------|
| Calcium phosphate and fluoride..... | 89.82 |
| Calcium carbonate..... | 4.37 |
| Magnesium phosphate..... | 1.34 |
| Other salts..... | 0.88 |
| Nitrogenous substance..... | 3.39 |
| Fat..... | 0.20 |

showing that the mineral content is normally very high.

Gies³⁷ has investigated the organic matter in the enamel of teeth and found that the protein present is keratinous in nature and does not yield gelatin with hot water. This is sufficient proof that enamel is epiblastic in origin.

The organic content of enamel is independent of the dentition or of the age of the teeth. Enamel is therefore different from other calcified tissues in origin and degree of calcification. The relation to its formative organ and the form of the structural elements of the tissue are also unique.

Dentine.—This substance is similar to bone in composition, but contains less water. On decalcification and subsequently boiling with water, it yields gelatin. Its structure, however, differs from that of bone.

Cementum.—This is identical with bone in structure and chemical composition. Both dentine and cementum are mesoblastic in origin. The latter covers the dentine in the root portion and in most cases overlaps the dentine at the gingival line.

Slight changes in the ratio of calcium, magnesium, carbonate, phosphate and fluorine have a great effect on teeth. A slight increase in carbonate or fluoride increases the hardness.*

The Chemistry of Ossification.—Bone and teeth, though usually containing a fairly high percentage of inorganic matter, are a living organized tissue in dynamic equilibrium with the tissue fluids. They are composed mainly of calcium phosphate, $\text{Ca}_3(\text{PO}_4)_2$.

Robison and his coworkers⁴⁰ found that the enzymes in tissue extracts hydrolyzed the soluble salts of hexose monophosphate, giving sparingly soluble calcium and barium phosphates. An enzyme capable of bringing about this change was found by them in bone, teeth and ossifying cartilage. According to Robison and his colleagues, this bone enzyme (phosphatase) rapidly hydrolyzes the phosphoric esters of glycerin and hexose present in blood, and the liberated phosphoric acid forms insoluble calcium phosphate with the calcium salts present in blood, which is deposited on the ossifying cartilage. The reaction takes place in a distinctly alkaline medium ($\text{pH} = 8.4$). This process may be viewed as an ordinary chemical reaction following the law of mass action—

$$\frac{[\text{Ca}']^3 \times [\text{PO}_4''']^2}{\text{Ca}_3(\text{PO}_4)_2} = K$$

$\text{Ca}_3(\text{PO}_4)_2$ has a very low solubility, so that $[\text{Ca}']^3 \times [\text{PO}_4''']^2 = K$.

The effect of changes in pH on the solubility product and of the presence of inorganic phosphates, glycerol, etc., on the activity of this enzyme have been studied.

This bone enzyme was found to be present in unossified cartilage only in very small amounts; and Robison concluded that the production of this enzyme is a part of those cellular activities which result in the formation of bone. The work has been extended to a study of the formation of the phosphatase in embryonic tissues and of bone development in embryo.

* For further information on the structure of teeth the reader is referred to the following references: ^{38. 39.}

It is maintained by some that the action of the bone esterase is to react with the organic phosphorus compounds in the blood plasma, thus increasing the concentration of the HPO_4 ions. The latter then react with calcium ions, depositing calcium phosphate as $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$. In the ossifying tissue this compound is rapidly converted into $\text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$.*

THE ABNORMAL CALCIFIED TISSUES OF VERTEBRATES

The calcified tissues of the animal body, as is well known, become abnormal through pathogenic conditions, unsuitable diet, or activity or inactivity of the endocrine organs. The first class hardly comes within the scope of this chapter and the reader is therefore referred to medical treatises on these subjects. The second and third classes which include such diseases as rickets, osteomalacia, osteoporosis, etc., of bone and caries of the teeth, will be briefly considered here.

Diseases of Bones.—The abnormal conditions mentioned above are those connected with general disturbance of nutrition. Rickets occurs in children and young animals and is characterized by (a) enlargements at the epiphyseal junctions; (b) softening and bending of the bones and their stunted growth; (c) abnormal proliferation of the epiphyseal cartilage; (d) formation of bonelike tissue deficient in calcium salts.

It has been found that this disease may be produced in a young growing animal by a deficiency of calcium, phosphate, or vitamins C and D in the diet. Provided that the intake of calcium and phosphate is normal, rickets may be cured by the addition of vitamin D to the diet or by the ultraviolet light treatment.

From the work on tetany which is associated with hypoparathyroidism, it has been found that, during an attack of tetany, calcium in blood serum is low; *i. e.*, 4–7 mg. instead of 10–11 mg. per 100 cc.^{44, 45, 46} It has been found that administration of calcium salts temporarily reduces the tetany, while parathyroid injections bring about normal conditions.

From the present knowledge of the relationship between vitamin D and the parathyroid and their effect on bone structure, it is considered that vitamin D controls the absorption and excretion of calcium through the intestinal wall,⁴⁸ whereas the parathyroid regulates the blood calcium⁴⁷ and probably stimulates the formation of osteoblasts and osteoclasts.⁵²

Harris⁴⁹ points out that the parathyroid is independent of vitamin D; the former raises the blood calcium by drawing it from the body.

It is said that the bone from a rachitic animal does not give a typical gelatin with hot water; which, if true, shows a degradation of the organic matrix of bone. The tests usually applied are confined

* For further information on the subject of ossification the reader is referred to the following references: ^{41, 42}

almost entirely to the determination of the ash content and to an examination by x-ray analytical methods.

Osteoporosis and osteomalacia are confined usually to adults. In the former, there is a lack of calcium salts, not affecting the hardness but making them thinner. Women usually suffer from the latter and the bones become soft and flexible, due to loss of calcium. This occurs most frequently during pregnancy owing to a diet lacking in calcium salts.

Osteitis Fibrosa.—This is a result of hyperparathyroidism, due to a tumor in the nature of adenoma. In this case, the calcium of the blood plasma may be raised from 12–17 mg. per 100 cc. Marble-bone disease is also a result of hyperparathyroidism.*

Diseases of Teeth.—The dietary diseases of teeth are the so-called "periodontal," including pyorrhea alveolaris. This is caused by a deficiency of vitamin A in the diet; and in extreme cases, absorption of the bone of the teeth takes place.

Hypoplasia.—This is seen in animals, particularly horses, where the diet contains the anticalcifying factor present in cereals with little vitamin D. This causes interglobular space in the dentine and poorly developed enamel.

Teeth which are hypoplastic are prone to *dental caries*. In caries, well-calcified secondary dentine is developed when there is plenty of vitamin D in the diet. This prevents the bacteria from eating further into the tooth. Vitamin D controls the calcification of alveolar bone and other calcified tissues. Recently there has been isolated a phosphatase from teeth similar to that found in bones.

That a deficiency of vitamin D in the diet is conducive to caries is not generally accepted.^{53, 54} Sampson⁵⁵ and Sprawson⁵⁶ support this theory, but Fish⁵⁷ is opposed to it.

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REFERENCES

1. Armstrong, E. F.: *The Simple Carbohydrates and Glucosides* (1924).
2. Abderhalden, E., and Straus, E.: *Z. physiol. Chem.*, **48**, 49, 535 (1906).
3. Kossel, A., and Kutscher, F.: *Z. physiol. Chem.*, **31**, 164 (1901).
4. Dakin, H. D.: *J. Biol. Chem.*, **44**, 499 (1920).
5. Abderhalden, A., and Voitinovici, A.: *Z. physiol. Chem.*, **52**, 348 (1907).
6. Abderhalden, A., and Schittenhelm, A.: *Z. physiol. Chem.*, **41**, 293 (1904).
7. Schwartz, H.: *Z. physiol. Chem.*, **18**, 486 (1894).
8. Kossel, A., and Kutscher, F.: *Z. physiol. Chem.*, **31**, 164 (1900–01).
9. Levene, P. A.: *Hexoseamines and Mucoproteins* (1925).
10. Morgulis, S.: *J. Biol. Chem.*, **93**, 455 (1931).
11. Green, E., and Tower: *U. S. Fish Commission Bull.*, **21** (1901).
12. Kay, H. D.: *Annual Rev. of Biochem.*, **1**, 187 (1932).
13. Gassmann, T.: *Z. physiol. Chem.*, **192**, 61 (1931).
14. Klement, R.: *Z. physiol. Chem.*, **184**, 132 (1929).
15. Shear, M. J., and Kramer, B.: *J. Biol. Chem.*, **79**, 105 (1928).
16. Taylor, N. W., and Sheard, C.: *J. Biol. Chem.*, **81**, 479 (1929).
17. Rosebury, H. H., Hastings, A. B., and Morse, J. K.: *J. Biol. Chem.*, **90**, 395 (1931).
18. Knaggs, J., and Schryver, S. B.: *Biochem. J.*, **18**, 1079, 1095, 1102 (1924).

* Other references to the literature are ^{50, 52, 48, 51}.

19. Knaggs, J.: *Biochem. J.*, **23**, 1308 (1929).
20. Northrop, J. H.: *J. Gen. Physiol.*, **3**, 715 (1921).
21. Hawk, P. B., and Gies, W. J.: *Am. J. Physiol.*, **5**, xv, 387 (1901).
22. Gies, W. J., et al.: *Am. J. Physiol.*, from 1901 on.
23. Alexander, J.: *Allen's Commercial Organic Analysis*, **10**, 119 (1933).
24. Dept. of Scientific and Industrial Research, London. Report in Colloid Chemistry, Nos. 1-5 (1917-23).
25. Reports on the Adhesive Research Com., Dep't. of Scientific and Industrial Research, London (1920; 1926; 1932).
26. Speakman, J. B.: *Proc. Roy. Soc. (London)*, **132A**, 167 (1931).
27. Astbury, W. T., and Street, A.: *Phil. Trans.*, **230A**, 75 (1931).
28. Speakman, J. B.: *Trans. Faraday Soc.*, **25**, 92, 169 (1929).
29. Speakman, J. B.: *Trans. Faraday Soc.*, **26**, 61 (1930).
30. Gerngross, O., et al.: *Naturwissenschaften*, **18**, 754 (1930).
31. Gerngross, O., et al.: *Ber.*, **63B**, 1603 (1930).
32. The Swelling of Proteins, Intern. Soc. of Leather Trades' Chemists (1933).
33. Hawk, P. B., and Gies, W. J.: *Am. J. Physiol.*, **7**, 340 (1902).
34. Marriott, R. H.: *J. Soc. Leather Trades Chem.*, **5**, 2, 280 (1921).
35. Noyes, B.: *A Text-Book of Dental Histology and Embryology* (1930).
36. Fish, E. W.: *An Experimental Investigation of Enamel, Dentine, and Dental Pulp* (1933).
37. Gies, W. J.: *J. Dental Res.*, **6**, 143 (1926).
38. Macintosh, J. F., Montelius, G., and Ma, Y. C.: *Chinese Med. J.* (1932).
39. McCollom, E. V., Simmonds, N., Becker, J. E., and Bunting, R. W.: *J. Biol. Chem.*, **63**, 547 (1925).
40. Robison, R.: *Biochem. J.*, **17**, 286 (1923).
41. Pryde, J.: *Recent Advances in Biochemistry* (1931).
42. Kay, H. D.: *Physiol. Rev.*, **12**, 384 (1932).
43. *Annual Rev. of Biochem.*, **2**, 207 (1933).
44. Thompson, D. L., and Collip, J. B.: *Physiol. Rev.*, **12**, 309 (1932).
45. Collip, J. B.: *Medicine*, **5**, 1 (1926).
46. MacCallum, and Vogel: *J. Exp. Med.*, **18** (1913).
47. Salvesen, H. A.: *J. Biol. Chem.*, **56**, 443 (1923).
48. Taylor, C. B., and Weld, H. D.: *Brit. J. Exp. Path.*, **13**, 109 (1932).
49. Harris, L. J.: *Lancet*, **1**, 1031 (1932).
50. Cameron, A. T.: *Recent Advances in Endocrinology* (1933).
51. Knaggs, R. L.: *The Inflammatory Diseases of Bone* (1926).
52. Selye, H.: *Endocrinology*, **16**, 547 (1932).
53. Mellanby, M.: *Brit. Med. J.*, **2**, 749 (1932).
54. Mellanby, M., and Pattison, C. L.: *Brit. Med. J.*, **1**, 507 (1932).
55. Sampson, W. E. A.: *Brit. Med. J.*, **1**, 534 (1932).
56. Sprawson, E.: *Brit. Med. J.*, **2**, 516 (1932).
57. Fish, E. W.: *Brit. Med. J.*, **2**, 747 (1932).

CHAPTER XXV

FUNCTION OF WATER IN THE ORGANISM

Water Content of Tissues.—Some idea as to the usual tissue content of water may be gained from the following table:

NORMAL WATER CONTENT OF THE TISSUES

| Tissue | Matthews | Long | Robertson |
|---------------------------|----------|------|---|
| Saliva..... | .. | 99.5 | |
| Cerebrospinal fluid..... | .. | 99.0 | |
| Vitreous humor..... | .. | 98.5 | |
| Embryonic brain..... | 91 | | |
| Milk..... | .. | 88.0 | |
| Brain (gray matter)..... | 84 | 86.0 | 82-85 |
| Kidney..... | 78 | 83.0 | |
| Thyroid..... | .. | | 77-82 |
| Thymus..... | 77 | | 81 |
| Suprarenal..... | 80 | | |
| Blood..... | .. | 79.0 | |
| Pancreas..... | .. | 78.0 | |
| Muscle..... | 73 | 75.0 | 75-78 |
| Spleen..... | .. | 76.0 | |
| Liver..... | 76 | 70.0 | |
| Skin..... | .. | 72.0 | |
| Brain (white matter)..... | 68 | | 68-70 |
| Tendon..... | .. | | 56-68 |
| Cartilage..... | 67 | | |
| Elastic tissue..... | .. | 50.0 | |
| Bones..... | .. | 50.0 | Vertebrae and ribs 16-44 Extremities and skull 14-22 |
| Fat..... | 6-10 | 20.0 | |
| Dentine..... | 10 | 10.0 | |

As Robertson observes, it will be noted that the tendency is for the proportion of water to be "highest in those tissues which are undergoing the most rapid metabolic changes." It is not possible at the present state of our knowledge to realize completely the part played by water in the organism. Obviously the water content of various tissues is not always constant. This is particularly true in relation to the skin, muscles and kidney, the seats of the water depots, in which the water content varies tremendously.

With age the amount of water in the body varies somewhat. The fetus contains a great deal of water, and the younger the fetus, the greater the amount. At birth, the amount of water in the organism is relatively small, judging from the amount of water in the blood. The amount of water begins to increase at about the age of six months, at which time aging begins. In very old age the tissues are tough and appear to be dry, but the content of water is then increased from 81.2 to 84.8 per cent instead of from 75 to 80 per cent.

Fat alters the percentage of water in the body. A. Magnus-Levy wrote: "Generally about 20 to 22 parts of protein are soaked in and swollen with 78 to 80 parts (four times as much) water. Fat enters into the interstices of the protoplasm as a dry and waterless mass, neither driving water out from the tissues nor bringing it into them."

W. Engels has studied the reserves of water of the body following intravenous injection of solution of sodium chloride. He found that all the tissues except bone became more watery. The muscles, skin and kidneys took up the greater amount of water, increasing the percentage of water they contain by 3.86, 3.23, and 3.83, respectively. The muscles, representing 40 per cent of the weight of the body, take up more than two thirds of any added water, but continue to act normally in spite of the added moisture. Greene and Rowntree¹ have found that more than 90 per cent of the added water in states of water intoxication in rats is found in the muscles and skin; and that that which is still present in the alimentary tract, for obvious reasons, is excluded from consideration.

The Constitution of Water.—As commonly accepted, the constitution of water is H_2O and the molecular weight 18. That this holds true only under limited and unusual conditions is well known. The percentage composition of water, two atoms of hydrogen to one of oxygen, was proved by Cavendish in 1781, although the true explanation of his results was not apparent until the experiments of Lavoisier in 1783.

The physical properties of water, especially its freezing and boiling points, do not coincide with those of a simple compound containing three atoms only. Its high critical temperature, cohesion, refractive index, and high surface tension all indicate that its formula is more complex than H_2O . That this is true is widely accepted, at least for temperatures lower than 400° C.

Practically all investigators are agreed that water is a multiple or

polymer of H_2O . Roentgen first suggested that all abnormal properties of water could be accounted for qualitatively by assuming liquid water to be a saturated solution of ice in some other form of water. From a study of surface tension energy, it was concluded that liquid water must be a polymer of H_2O . Ramsay and Shields computed from the surface tension that the formula at the boiling point must be $(\text{H}_2\text{O})_3$, and in ice $(\text{H}_2\text{O})_4$. Determinations of the freezing point of solutions of water in other solvents point to the formula $(\text{H}_2\text{O})_2$. Sutherland has attempted to prove that steam is represented by H_2O , termed hydrol, and ice by $(\text{H}_2\text{O})_3$, trihydrol, liquid water being $(\text{H}_2\text{O})_2$, a mixture of trihydrol and dihydrol.

Relative to the chemical constituents of water, an interesting question now arises as to whether heavy water is ever encountered in the organism. Heavy water is water containing a heavy isotope of hydrogen. Such water has an increased viscosity, lower refractive index, lower surface density and in its salts are less soluble. Swingle and his collaborators have shown that it is toxic in concentrations greater than 30 per cent for tadpoles (*rana clamitans* and *lofius*), green frogs (*euglena viridans*), paramecia (*paramecia codatum*), common aquarium fish (*libistes reticulatus*) and the flat worm (*planaria* and *maculata*). Urey, Brickwedde and Murphy² have suggested the name *deuterium* for the heavy isotope and *protium* for the light isotope. They claim that there are at least three varieties of water and suggest the possibility of nine varieties. Obviously, there is much yet to be learned concerning the chemistry of water.

State of Water in the Organism.—Water exists in the organism mainly as a solution of salts, as a liquid phase of colloidal solution and as water of hydration. It is of vital importance, since its properties, and those of the cell, determine the amount of the various substances present and amounts in which they are exchanged. The turgor of cells is dependent on their content of water, which in turn is dependent on osmotic pressure or the capacity for hydration of protoplasm itself.

The crystalloids in the saline solution of the body exist in rather fixed concentration, much as in Ringer's solution, which contains sodium chloride 0.7 per cent, potassium chloride 0.03 per cent and calcium chloride 0.025 per cent. Locke's solution contains, in addition, sodium bicarbonate 0.01 to 0.03 per cent and glucose 0.1 per cent. Locke's solution approaches in composition deproteinized plasma and contains both electrolytes and nonelectrolytes.

Blood plasma is composed of water and salts, as is Locke's solution, and in addition, serum albumin, serum globulin, fibrinogen, gases, metabolites, enzymes, special substances such as hormones and so forth. Of the proteins, albumin forms somewhat more than 50 per cent, globulin somewhat less than 50 per cent and fibrinogen from 5 to 10 per cent.

General Function of Water in the Organism.—In the organism

water fulfills many functions. During its ingestion, if taken with food, it aids in its maceration, passage to the stomach and digestion in the stomach and gastro-intestinal tract and assimilated into the blood stream. It circulates in the blood or lymph as the universal solvent; it plays a part in many of the numerous hydrolytic or oxidative reactions; it makes up a large part of the tissues as a liquid phase of colloidal solution or water of hydration; it serves as a vehicle of transportation; it is excreted by one organ, only to be carried elsewhere and used in another field of chemical activity; it plays one of the leading parts in distribution and regulation of heat, and it acts as a lubricant on surfaces subject to friction. It reaches every cell in the organism, and through its properties furnishes the opportunity for chemical reactions, for changes in physical state and for transformation of energy.

After it has performed these functions, it continues to serve the organism, even during its excretion by way of the kidneys, bowels, lungs or skin, and it appears finally in the urine and feces, visible or invisible perspiration, or in the expired air. The relative amounts excreted by these different channels vary greatly from time to time in the same person even in health; all depends on the influence of almost innumerable factors.³

Solvent Properties of Water.—In the organism, water is the universal solvent, but even the process of solution of one salt in water is not well understood. It is evident, however, that dissociation occurs, and that in solution the molecules of a substance are free to manifest the effect of energy due to their movements. The process of solution is one of dispersion, similar in kind to that which occurs in colloid solutions but differing in the degree of subdivision and fineness of the disperoids.

When one considers the number and character of electrolytes, non-electrolytes, colloids and gases, which occur in the blood and urine, the tremendous efficiency of water as a solvent becomes apparent. Although all these substances are freely soluble in water, the majority of them are not soluble in other liquids, not even in alcohol.

Intermediate Water Exchange or Cyclic Use of Water.—Only one phase of the subject of intermediate water exchange is presented by way of illustration. Much fluid is absorbed from the digestive tract, but much, after absorption, finds its way back into the alimentary tract, there to help in the process of digestion. In fact, the quantity which enters the intestines, as digestive fluids, far exceeds that taken by mouth. The amount of fluid used in this way is large. In a man of average size, the quantity of water which enters the intestine as digestive fluid runs from 7500 to 10,000 cc. This is two to three times the amount of fluid ordinarily ingested by mouth, from three to four times the amount ordinarily excreted as urine and about twice as much as the total volume of the blood. As the contents of the intestine pass downward, most of this fluid is reabsorbed, so that the amount which remains in the feces rarely exceeds 200 cc. each day.

Water and the Plasma Proteins.*—By virtue of the hydrophyllic properties of proteins, their level in the plasma is of vital importance to the water content of the blood; so important, indeed, that the total plasma protein content (6.5 to 7) is now beginning to be regarded as one of the fixed bodily constants. The proteins concerned are serum albumin, serum globulin and a special globulin concerned in blood clotting, fibrinogen. The serum albumin usually comprises 4 to 5 per cent, an average of 4.4 per cent; serum globulin 2 to 3 per cent, an average of 2.6 per cent, and fibrinogen 0.2 to 0.4 per cent, an average of 0.3 per cent. The normal albumin globulin ratio is 1.72. With these values such plasma has a specific gravity approximating 1.027 and the proteins present exert an osmotic pressure of about 30 mm. Hg.

These plasma proteins are normally retained rather rigidly in the blood and though the osmotic pressure they exert (30 mm. Hg) is small compared with that exerted by plasma as a whole, it is a constant or fixed intravascular pressure and hence an important controlling factor in the maintenance of the fluid balance between the blood on the one side and the tissues, intercellular space and body cavities on the other. Because of the presence of these proteins and the influences they exert, the plasma should no longer be regarded as merely a vehicle for red blood cells but as a crucial participant in all processes that pertain to water metabolism and its clinical states within the organism.

Serum albumin constitutes the bulk of the proteins found in the plasma. It has the longest and heaviest molecule and exerts per gram about four times as great an osmotic pressure as does globulin. Hence its level in the blood is of the most vital significance. It is subject in disease to greater fluctuation and is more readily lost from the body and more slowly replaced if lost. Under great demand it can be regenerated in amounts up to 25 Gm. per day. Its source, seat of origin, is unknown as is also that of serum globulin. Fibrinogen on the other hand is known to come from the liver. Though albumin has the largest molecule, it diffuses more readily through the diseased kidney. Hence, deficiency of serum albumin is a frequent finding in nephritis and nephrosis characterized by marked albuminuria and renal dropsy. In such conditions there may be at times some compensatory increase in serum globulin.

In the modern tendency to emphasize the rôle of the colloids in the pathogenesis of edema, sight should not be lost of the importance of salts, especially of the chlorides and the sodium ions.

Water as a Vehicle of Transportation.—The solid tissues and organs of the body are served, nourished and cleansed by a water system. In addition, through washing down of food by water, the waste products of the body are removed by water. So far as the cell is concerned, blood brings it its food and drink and the lymph and blood carry off the excess and noxious products resulting from metabolism.

*See also Chapter XV.

The blood, however, constitutes the great internal water system. Its relation to gaseous metabolism has always been appreciated. The plasma has usually been regarded merely as the vehicle for the transportation of the erythrocytes. Obviously, this overlooks its true significance. What the erythrocyte is to gaseous metabolism, the plasma is to nitrogen, mineral and water metabolism. The plasma supplies the fuel; the erythrocytes the oxygen needed for internal combustion. In a sense, the plasma itself constitutes a kind of liquid fuel.

Many have recognized that the plasma should be regarded as a moving tissue, which circulates throughout the body and which by virtue of its physical properties is permitted unbounded opportunity for chemical activity. As such it becomes one of the most interesting tissues of the body. Plasma is adapted to the needs of every tissue and cell of the body. It distributes food (glucose, amino acids and lipoids). It carries salts in balanced amounts to maintain osmotic relationships and it furnishes water as a medium for chemical reactions. It maintains itself in constant acid base equilibrium and acts also as a buffer. It is, also, itself, the seat of chemical reactions and participates in innumerable reactions. It transports hormones and carries the specific products of cells from the seat of origin to their allotted seat of function. It conveys enzymes which work in both the blood and tissues and it collects and removes waste products, conducting them to the appropriate organ of excretion. It gets into and out of capillaries and into and out of individual cells. Within itself it carries the materials necessary for protection and repair, immediately and automatically, sealing rupture of the walls of the channels in which it circulates. It participates in the production, distribution and dissipation of heat. It maintains its volume and composition constant despite innumerable small increments and losses. The plasma, therefore, is a vehicle of transportation which in health continuously replenishes and purifies itself and maintains itself constant in volume and composition as it circulates to meet the needs of the body as a whole.

Water and Heat Regulation.—Water regulates the temperature of the environment and the organism. This is made possible through its unique thermal properties. The specific heat of water is the highest known for any substance, solid or liquid, with one exception. The latent heat of vaporization of water is the highest known, while the latent heat of cooling, is next only to that of ammonia. The latent heat of vaporization is of universal significance in relation to dissipation of body heat because evaporation occurs at all temperatures. The amount of vapor that air can hold when in contact with liquid is variable, is dependent on temperature and pressure, and is greatest for fluids whose latent heat of vaporization is greatest. Consequently more water can vaporize than any other substance. Heat conduction of water, although low as compared with that of some metals, is yet

the greatest known for any liquid. Hence water serves best of all liquids in heat conduction in and away from the body. It is the most ideal buffer for heat in existence; that is, it exerts unparalleled resistance to heat or cold before changing its temperature or its physical state, while in addition its high latent heat of vaporization is of constant value to the organism in the dissipation of heat.

Water, by virtue of mobility, is readily shifted so as to meet the constantly changing needs in relation to heat regulation in the body. By virtue of its stability, it places its thermal properties at the disposal of the organism for this important function. Besides, of all liquids, it is the most available.

Water, then, regulates heat distribution and dissipation through its mobility and its ideal thermal properties: (a) High specific heat, which favors storage; (b) high caloric demands for its evaporation, which permit a rapid elimination of heat; and (c) high heat conductivity which provides rapid equalization of heat within the tissues of the body.⁴ The regulating mechanisms in regard to its dissipation are: (a) The vasoconstrictor center and the vasoconstrictor fibers in the skin; (b) sweat and the sweat centers and nerves; and (c) the respiratory center. The nervous mechanism is responsible for the mammal being homeothermic rather than poikilothermic, and destruction of this mechanism results in its conversion from the former to the latter state.

Lubrication.—Nature's method of water lubrication has never been approached or excelled. In millions of bodies pleura has rubbed against pleura, pericardium against pericardium and intestine against intestine, constantly and continually, day in day out for sixty, seventy or eighty years without mishap and without pain. A hundred or two hundred joints in the body have flexed and extended, synovia rubbing on synovia often under considerable pressure, without injury and without discomfort.

The mechanism involved in this lubrication has never been properly investigated from either the chemical or physical side. In the lubrication of joints water is the chief consideration. Grease and oil are missing since the lipid content of synovial fluid is but a small fraction of 1 per cent. It is known that a mucus-like substance, synovin, is present in small amounts, but its exact nature is not as yet determined. In the body water is the lubricant *par excellence*.

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REFERENCES

1. Greene, C. H., and Rowntree, L. G.: *Am. J. Physiol.*, **80**, 209 (1927).
2. Urey, H. C., Buickwedde, F. G., and Murphy, G. M.: *Science*, **78**, 602 (1933).
3. Adolph, E. F.: *Physiol. Rev.*, **13**, 336 (1933).
4. Barbour, H. G.: *Physiol. Rev.*, **1**, 295 (1921).
5. Henderson, L. J.: *The Fitness of the Environment* (1913).
6. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry* (1931).

CHAPTER XXVI

THE CHEMISTRY OF MUSCLE

To describe an automobile in terms of the percentage of wood, gasoline, iron, etc., in the entire mechanism would convey very little information as to how automobiles work; a few inferences could be drawn, particularly from comparisons of the analyses of different kinds of cars, and of cars before and after spells of work. The function of the 5 per cent or so of volatile hydrocarbons could be fairly quickly ascertained. The evolution of hydrogen gas from the minced automobile and the presence of copper sulphate in the autolyzed residue might suggest to a thoughtful chemist the presence of free sulfuric acid in some part of the original mechanism—acid restrained by some impermeable membrane from attacking the metallic constituents *in vivo*.

Analysis of Muscle.—The analysis of various kinds of muscle produces information of a similar degree of indirectness, most of which we cannot interpret at all. The following table contains the analytical

THE APPROXIMATE COMPOSITION OF A RESTING SKELETAL
MUSCLE OF A FROG

| | |
|--|-------|
| Water | 80 |
| Protein | 17 |
| Fat, and lipids | 0.2 |
| Glycogen | 0.7 |
| Hexose monophosphoric ester | 0.05 |
| Creatinephosphoric acid | 0.45 |
| Lactic acid | 0.015 |
| Carnosine | 0.25 |
| Creatine | 0.08 |
| Adenosine triphosphoric acid (adenylpyrophosphate) | 0.25 |
| Urea | 0.01 |
| Orthophosphate (as H_2PO_4) | 0.045 |
| Chloride | 0.05 |
| Bicarbonate | 0.03 |

About 5 per cent of the phosphorus of the muscle is not accounted for by the substances in this list, and about 10 per cent of the non-protein nitrogen. The sodium content of muscle is approximately equivalent to the chloride and bicarbonate, and the potassium is about equivalent to the total phosphate, free and conjugated.

figures of a resting skeletal muscle from a frog. The figures do not add up to 100 per cent for we do not know all the substances present, and nobody has analyzed a single muscle for all the known substances. From outside evidence we know that some of the protein is of structural value. The voluntary muscles of other vertebrates give similar results, though there are some characteristic differences in quantitative relationships. Thus the muscles of active animals contain, on the whole, more creatine, free and combined, than those of sluggish animals, and even in one and the same animal the more rapid muscles

contain more than the others. Again, some vertebrates (cat, rabbit) have little or no carnosine in their muscles, but have instead methyl-carnosine (anserine¹). The muscles of invertebrates, on the other hand, contain neither creatine² nor carnosine,³ but they contain arginine, both free and in combination with phosphoric acid.⁴ This arginine seems to be the invertebrate analogue, physiologically speaking, of the creatine of vertebrate muscles, and it is important that non-muscular tissues (other than nerve) do not contain significant amounts of creatine or arginine. Carnosine and anserine are peculiar to muscle tissue also, and presumably have some part to play in the special function of muscle, but no good experimental evidence has been put forward which gives any suggestion as to what this function is.

Muscle in the Resting State.—Analyses of different sorts of muscle give some hint therefore as to which constituents are likely to have special relation to the function of muscle, and some more definite information is yielded by analyses of muscles in the resting and rapidly exhausted states. Such comparisons reveal that some of the glycogen has disappeared, and most of the creatinephosphoric acid: In their place, and accounting quantitatively for them, are increased amounts of hexosephosphate, lactate, phosphate and creatine. No other changes of any magnitude are consistently found to accompany fatigue. Some other effects have been observed sometimes, but unlike those just noted, they are not reversed by subsequent recovery of the muscle in oxygen, and are therefore probably death changes, or the results of local injury.

Composition of Muscle.—Before studying these changes in detail it is well to attempt to form a more exact mental image of the composition of the muscle in relation to its structure. Even if we neglect structures such as blood vessels, nerves and nerve endings, and sinew, the muscle substance proper is still composed of cells with fluid between; and it is to be expected that these two "phases" should differ in composition, the latter resembling, we should expect, blood plasma. And the microscopic appearance of a single fiber prepares us to find that it is highly heterogeneous in chemical composition. The unit structure of the voluntary muscle is the sarcomere, the segment of a fibril lying between two consecutive Z membranes; and ultimately biochemical knowledge of muscular contraction has to be related to the structural changes occurring within this motor unit.

Very little satisfactory information is, however, available to help us to picture the distribution of substances in the tissue. When an isolated frog muscle is immersed in isotonic sugar solution the sodium and chloride in it diffuse away rapidly and almost, if not quite, completely. Only a small part of the phosphate is lost, however, and practically none of the potassium. The sodium chloride seems, from the evidence, to be contained between the cells, rather than in them, in distinction from the potassium salts. Again, although such a muscle loses none of its carnosine when kept in Ringer's solution, yet addition of carnosine to the solution results in the immediate penetration of the

muscle by carnosine up to a limit which can be expressed as 30 per cent of the water of the tissue, if we assume simple osmotic pressure to be the governing consideration. This carnosine can diffuse out again if the muscle is put into fresh Ringer's solution. These observations suggest that the carnosine of the muscle is retained within the cells or within parts of each cell, totaling 70 per cent of the muscle water.⁵

Urea and histidine can penetrate reversibly the whole of the living muscle substance. When a muscle has reached equilibrium with a saline containing either of these substances, the concentration in the tissue is 80 per cent of its concentration in the saline, which would be expected to result from simple diffusion equilibrium since the muscle contains 80 per cent of water.⁶ Only in respect to such substances as these can the chemist's analytical figures be taken to have a literal physical meaning.

Attempts have been made to use histological staining technics to discover in what region of the tissue certain solutes are contained. All such work must be done with the utmost care to prevent postmortem changes, else they are meaningless. Dead muscles are quite homogeneous and freely permeable, for example, towards carnosine, differing markedly from the living. It has been possible to show by a staining reaction that phosphate is not evenly distributed through the muscles of certain insects. Yet another technic has been used, which, however, suffers from the same disadvantage of exposure to the risks of post-mortem changes; this consists in rapid ashing of the intact isolated muscle. The ash residues are found to preserve the structural details of the tissue with remarkable fidelity, and reveal a decided localization of the inorganic constituents in the dark bands (Q disks), which occupy the central portion of each sarcomere, and in the Z membranes, which separate successive sarcomeres.

Osmotic Pressure of Muscle.—The unequal distribution in the living tissue of substances known to be diffusible *in vitro* raises difficulties in the interpretation of the vapor pressure depression of the water of the muscle. The vapor pressure of resting muscles of the frog can be measured by the thermometric technic of A. V. Hill and proves to be approximately that of a 0.72 per cent solution of sodium chloride.⁷ This is presumably the vapor pressure of the fluid lying between the cells, which is probably of quite different composition from the cell contents, and the measurements tell us about the osmotic pressure of the cell fluid only if we make assumptions as to what state of strain, electrical or mechanical, exists in the membranes separating cell from interspace. It may be possible in time to measure the partial osmotic pressure of each of the diffusible substances in the intercellular fluid by the method of counterdiffusion already applied to creatine and phosphate. The former is found to be present in a concentration of about 80 mg. per 100 Gm. and the latter about 10 mg. per 100 Gm. in the intercellular spaces of the resting muscles, and the figures are about

doubled by fatigue. The apparent concentration of lactate (measured by counterdiffusion) rises similarly in fatigue from about 29 mg. to about 240 mg. per 100 Gm.⁸

The total osmotic pressure of the muscle rises, as Hill showed, closely parallel with the energy output of the muscle measured by the heat developed anaerobically in a series of twitches. Both the heat production and the osmotic pressure increase are therefore fairly directly proportional to the tension development; for the simple relation of the heat production to the tension production was established several years ago. More briefly, it can be stated that to a fair degree of approximation the following relationship holds for isometric twitches up to moderate fatigue:

Total Tension developed \propto Heat liberated \propto Osmotic Pressure Increase.

Each of these aspects of activity is a measure of the total chemical change occurring in the muscle; and the simple relationship between them therefore develops special interest in view of the fact that neither of the two major chemical accompaniments of fatigue is found to be proportional in extent to the energy output. These reactions are the hydrolysis of creatinephosphoric acid and the formation of lactic acid. The former occurs rapidly in the initial twitches of a series and falls off practically to zero at the end.⁹ Of the latter little, if any, is produced in the first few twitches and the rate of production increases as fatigue advances.¹⁰ Viewed as sources of osmotic pressure increase, the two reactions taken together are more nearly proportional in extent to the degree of fatigue; but actually the osmotic pressure rise (as measured by the fall in vapor pressure) is considerably greater than can be accounted for by these reactions alone. Other reactions resulting in osmotic pressure increase must occur to an extent about comparable to these two, if the observed osmotic pressure increase is to be accounted for. The following imaginary case illustrates quantitatively the difficulty. It is a matter of experience that a frog sartorius weighing 0.2 Gm. and having a length of 4 cm. would produce a total tension in a series of isomeric twitches (anaerobically) of about 10 Kg. before exhaustion sets in. The heat evolution would be about 0.16 calorie and the osmotic pressure increase would be equivalent to 0.32 per cent NaCl. But the increase in lactate concentration (0.25 per cent) would account for only a quarter of the osmotic pressure increase (even assuming the precursor to have a negligible osmotic pressure) and the phosphagen breakdown (0.07 per cent in terms of its P content) would account for only one fifth. There would remain rather more than half of the total change to be accounted for in other ways. The fact that the reactions almost certainly occur within the cells, while the osmotic pressure measurements refer to the fluid surrounding them, does not lessen the difficulty, for the rise in the osmotic pressure of the cell contents must be greater than the observed change in the intercellular fluid, since the fibers tend to swell.

If a muscle be deprived of oxygen but not stimulated into activity there occur in the muscle the same chemical breakdowns as are induced by fatigue, to the same extent in the end (although about 250 times more slowly), and in the same order: That is, phosphagen breakdown is rapid at first and slows up as the residue becomes smaller while lactate production tends, if anything, to accelerate (Fig. 45). Ultimately the oxygen lack leads to irreversible breakdown of other substances and eventually to a contracture identical with rigor mortis. Now there is no obvious necessity for the relaxed muscle to use up the chemical compounds which have been stored in it especially for the fulfilment of its contractile function; and this observation sug-

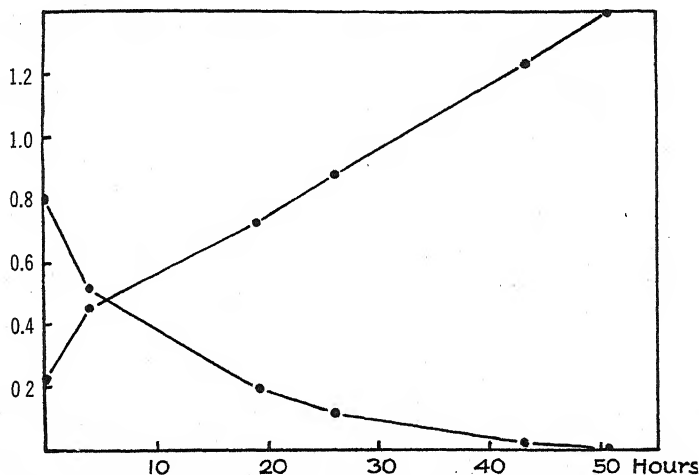


Fig. 45.—The phosphagen and orthophosphate contents of frog skeletal muscle resting in nitrogen at room temperature. (From analytical figures given in Eggleton and Eggleton.⁴²)

Ordinates: Inorganic and phosphagen phosphorus as fractions of their combined value at the start.

Abscissae: Duration of anaerobiosis.

Only after thirty hours does phosphate appear from sources other than the hydrolysis of creatinephosphoric acid.

gests therefore the possibility that these breakdown reactions are necessary, not to produce contraction, but to bring about, and even to maintain, relaxation. This view was put forward by A. D. Ritchie on other evidence¹¹ and will be discussed again later.

Stages of Fatigue.—It is convenient at this point to take up the question why the earlier stages of fatigue are characterized by a relatively high phosphagen breakdown and low lactate production, while in the later stages the situation is reversed. Both of these reactions result in the liberation of energy, and can indeed account for all the energy released by the muscle during its activity. But it is not necessary to suppose that the muscle has two mechanisms for producing energy and changes over from the one to the other. The solution of

this problem was provided by Lundsgaard¹² who showed that muscles poisoned with iodoacetate produce no lactate during anaerobic activity, but lose their phosphagen to *an extent strictly proportional to the energy output even up to extreme fatigue*. When their phosphagen content is used up the muscles go into a state of contracture (Fig. 46).

Lundsgaard showed further that the hydrolysis of the phosphagen was just sufficient to account for the energy output of the muscle, if Meyerhof's figure for the heat of hydrolysis be accepted. This, however, must not be stressed too highly, for the heat of hydrolysis has not been measured with a high degree of accuracy; moreover, the reaction occurring in these muscles is not a simple hydrolysis, for no inorganic phosphate is produced. Instead, there accumulates an equivalent amount of hexosephosphoric esters. Again, Hill has shown that there occur in these poisoned muscles unknown chemical reactions producing an osmotic pressure increase equal in extent to that of the normal

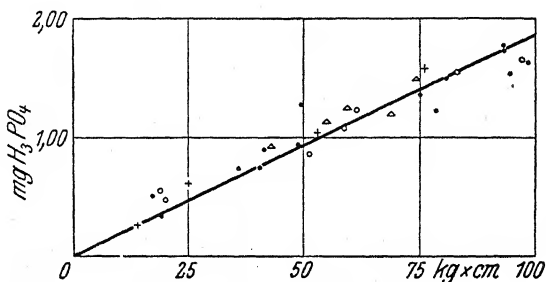


Fig. 46.—The phosphagen breakdown resulting from different degrees of fatigue (isometric twitches) in frog gastrocnemius muscle poisoned with iodoacetate. Some of the muscles were also curarized. (From Lundsgaard.)

Abscissae: Sum of the tensions registered in the twitches, multiplied by the length of the muscle.

muscles¹³ and these hydrolytic reactions are likely to produce some heat.

Nevertheless, Lundsgaard's demonstration seems to be of supreme importance in indicating that the function of the reactions leading to the production of lactic acid is not concerned with contraction or with relaxation, but appears to make possible a partial restoration in anaerobic circumstances of the phosphagen lost in activity, thereby delaying considerably the onset of exhaustion; in the case of frog muscle, indeed, more than doubling the amount of activity possible in the absence of oxygen.

Although Lundsgaard has shown that a close relationship exists between phosphagen breakdown and activity, it still remains an open question whether this breakdown produces the contraction or, as Ritchie has suggested, the relaxation. The question will probably never be decided by direct chemical analysis, for the change involved in a single twitch is too small to be measured (about 0.01 mg. of phos-

phagen P per Gm. of muscle); and, moreover, it is difficult if not impossible to kill a muscle in the relaxed state. The time relationships of the different phases of heat production associated with the twitch are of interest in this connection, although they do not afford a definite answer to the question. By cooling muscles to $0^{\circ}\text{C}.$, and thereby slowing up the changes occurring in a twitch, Hartree¹⁴ has been able to show that the heat liberated at the moment of the twitch (a quantity referred to in the literature as the "initial heat," which is the same in amount whether oxygen is present or not), is set free in two quite distinct batches: One during the establishment of tension, and the second, half as large, during relaxation (Fig. 47). The examination of muscles poisoned with iodoacetate in similar experiments has led to another

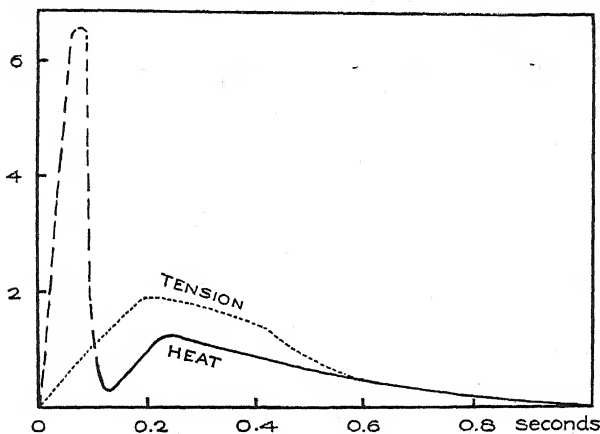


Fig. 47.—The time-course of heat production rate and tension development in an isometric twitch of a frog sartorius muscle at $0^{\circ}\text{C}.$ The exact shape of the earlier part of the heat curve is uncertain. (Redrawn from Hartree.¹⁴)

Ordinates: Heat production rate as a multiple of the mean value for the first second. The ordinates for the tension production curve are not given.

Abcissae: Time in seconds.

result of far-reaching importance, namely that no difference of any kind can be observed between poisoned and unpoisoned muscles in respect of these measurements. In the poisoned muscle no lactate or inorganic phosphate has been produced, yet the total heat and its distribution in time are unaltered. The only thing known to be in common between the two cases is the disappearance of creatinephosphoric acid, and if any correlation can be made at all, it must be between this phosphagen disappearance and the heat evolution.

The view put forward by Ritchie harmonizes with this distribution of the initial heat if we suppose that the second batch of initial heat (the "relaxation heat") represents a wasted fraction of the heat of hydrolysis of phosphagen, the remainder being used to bring about relaxation, and being stored therefore as potential energy in the re-

laxed muscle. This energy is released as the first batch of heat (the "contraction heat") in the next twitch.

During the half minute or so following a twitch or a series of twitches, there is a small and rather variable heat evolution known as the "delayed anaerobic heat," and during this period there occurs (as was shown by Gorodissky and by Nachmannsohn) a reappearance of part of the phosphagen lost during activity (Fig. 48), and it has further been established during the last few years that most, if not all, the lactic acid production associated with the activity occurs during this period also, and not, as was earlier thought, during the twitch.^{15, 16, 17} The lactic acid production is exothermic, while the phosphagen resynthesis is endothermic, and as the extents to which they occur are

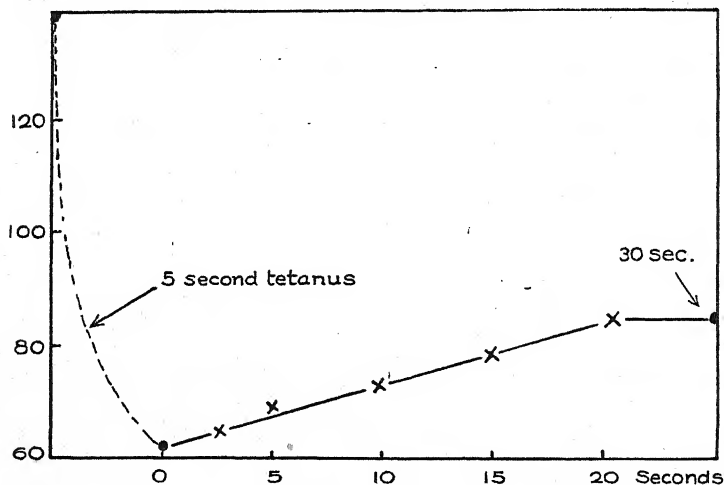


Fig. 48.—The reappearance in the course of twenty seconds, of part of the phosphagen lost during a five-second tetanus, the conditions being strictly anaerobic. (Redrawn from Nachmannsohn.⁴²)

Ordinates: Phosphagen content of muscle in mg. of P_2O_5 per 100 Gm.

variable it is easy to agree with Hartree that the delayed anaerobic heat is probably the small and variable balance between the two processes. It may be remarked in passing that on this view there should be no delayed anaerobic heat from muscles poisoned with iodoacetate, since in these muscles neither lactic acid production nor anaerobic phosphagen resynthesis occurs.

Admission of Oxygen to Muscle.—If oxygen is admitted to the muscle after a short spell of activity there occurs a further evolution of heat spread over a few minutes and roughly equal in extent to the initial heat; and during this time there occur (a) the resynthesis of the phosphagen broken down; (b) the removal of the accumulated lactate, and the reappearance of part of the glycogen lost; (c) the absorption of oxygen and the liberation of carbon dioxide; and (d) the reversal

of the osmotic pressure changes. The small accumulation of hexose-monophosphate which occurs in a normal frog muscle during activity does not appear to be readily reversed, though a reversal during oxidative recovery has been demonstrated clearly with certain mammalian muscles.¹⁸

Comparison of these different accompaniments of oxidative recovery in a quantitative manner has, on the whole, led to disappointingly few results. For example, the ratio of carbon dioxide liberated to oxygen absorbed leads to figures for the respiratory quotient which, though tending to be high, do not indicate the exclusive use of carbohydrate as a fuel;¹⁹ and in this respect the results are in substantial agreement with those of investigations of exercise in intact mammals and in man.

Oxygen Consumption and Lactic Acid Disappearance.—Comparison of the amount of lactic acid disappearing with the amount of oxygen used demonstrates a not very strict relationship though it may be said that on the average 6 or 7 mg. of lactic acid are removed for every cc. of oxygen absorbed, or about five times as much as could be burned with the aid of this oxygen. It is not, however, necessary to suppose that any of the lactic acid is burned but simpler to suppose that all of the lactic acid is re-formed into its precursor and that a suitable amount of any available fuel is burned to provide the necessary energy. The quantitative relationship of phosphagen resynthesis to oxygen consumption was studied in frog muscles at low temperature (-0.5°C.) by Meyerhof and Nachmannsohn⁴¹ who observed that as an average for the complete oxidative restitution, 1 molecule of oxygen was absorbed for every 5 molecules of phosphagen resynthesized. Taking the calorific value of oxygen as approximately 5 Calories per liter (a figure practically independent of the nature of the foodstuff oxidized), this implies that about half the energy derived from the oxidations was stored in the form of resynthesized phosphagen. At higher temperatures the efficiency was less.

The comparison of glycogen reappearance with any of the other concurrent phenomena does not appear to have been attempted though the actual resynthesis of glycogen in the isolated muscle has been shown by Meyerhof and his colleagues, and its resynthesis in the muscles of the intact mammals recovering from exercise has been the subject of a considerable amount of work. In the case of the intact animal it has not been found possible to demonstrate any correlation, either in the quantities involved or in the time scale, between glycogen resynthesis and oxygen consumption or lactate removal.

The Chemical Basis of Muscular Contraction.—To summarize the view which has been put forward as to the chemical basis of muscular contraction, it seems likely that energy is stored in a relaxed muscle in some physical system which is maintained in its high potential state by the continued breakdown of phosphagen. In the absence of oxygen this breakdown can be partially reversed by means of the energy of

glycolysis; in the presence of oxygen it can be completely reversed, and a dynamic equilibrium attained by the energy of combustion of any foodstuff available. On this view the reestablishment of the relaxed state after a contraction involves exactly the same processes, greatly accelerated in speed. It must be clearly understood, however, that the evidence at present available permits equally the view that the resting muscle is in its low potential state, and that the breakdown of phosphagen occurs before the twitch, supplying directly the energy of contraction. The rapid loss of phosphagen from a resting muscle deprived of oxygen is ascribable on this view to an imperfection in the organization of the tissue, which does not in life meet complete oxygen lack in the resting state.

Heart Muscle of the Frog.—The chemical basis of the activity of the heart muscle of the frog seems to be similar to that of skeletal muscle, though the heart differs markedly from the latter in certain quantitative respects. Its resting metabolic rate is considerably higher than that of skeletal muscle, and the utmost activity to which it can be excited results in only a four- or fivefold increase in this metabolic rate, instead of the two hundredfold increase of which a skeletal muscle is capable. The relatively small phosphagen content of the cardiac muscle is probably connected with this fact; for, small though it is, the phosphagen content of the normal heart is several times larger than would be needed to energize a single beat from its heat of hydrolysis. The oxygen consumption and carbon dioxide output of the isolated heart, taking into consideration the small but persistent evolution of ammonia and urea, suggest that not more than two thirds of the fuel burned is carbohydrate and that the rest must be largely protein, since it has not been found possible to demonstrate the disappearance of fat.^{20, 21}

Deprived of oxygen, the heart can continue to function for hours provided the circulating fluid is kept alkaline. During this time lactic acid is formed steadily, and the heart apparently ceases to function only when all the available carbohydrate has been converted to lactate. This conclusion is suggested by the fact that when the heart fails in these circumstances it can be readily restored by the administration of glucose.²² Such an exhausted heart is not by any means carbohydrate-free, but it must be supposed that the residual carbohydrate is not available. During this prolonged period of oxygen lack, the phosphagen content of the heart remains at a very low level, but never disappears entirely.²³ This small amount is, on the view expounded in the earlier section, being continually broken down and reconstituted by the energy of glycolysis. That glycolysis is the only source of energy available to the anaerobic heart for this purpose is suggested by the observation that the anaerobic heart is brought rapidly to a standstill by the addition of iodoacetate to its perfusion fluid, and is then found to contain no phosphagen. In the presence of oxygen, a similar treatment with iodoacetate produces no effect demon-

strable within an hour or two, although if the oxygen is removed at any time during this period the heart ceases to beat within a minute or two and can be brought back into activity only by the immediate readministration of oxygen²³ (Fig. 49).

Production of Lactate from Glycogen.—The chemical mechanism bringing about the production of lactate from glycogen in the muscle cannot be studied very profitably in the intact tissue. Apart from small but suggestive fluctuations in the hexosemonophosphate content of the muscle and the presence in it of small traces of pyruvic acid and aldehyde, the glycolysis mechanism keeps itself so well balanced that only the initial and final states are demonstrable. Fortunately, the glycolytic function is displayed even by cell-free muscle extracts, in which it is possible by various treatments to injure the system at different points and bring about the accumulation of products, supposed to be intermediates, which do not normally accumulate. A quite fresh extract can produce lactate from glucose, hexosephosphates, glycogen or starch; but after aging a few hours at room temperature, it ceases

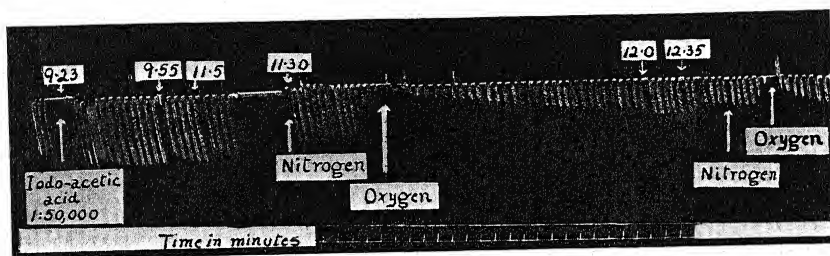


Fig. 49.—Showing that the primary effect of iodoacetate on the frog's ventricle is upon some substance or mechanism required by the muscle only when oxygen is lacking. (From Clark, Eggleton, and Eggleton.²³)

to be able to attack glucose. Dialysis of an extract renders it unable to glycolize anything but hexosediphosphate, but the extract can be reactivated by adding back the dialyzed material. A great deal of research, chiefly in Meyerhof's laboratory, has led to the identification of the essential constituents of the dialysate, and they prove to be magnesium, phosphate and adenylic acid,^{24, 25} but not glutathione.²⁶ This exception is particularly interesting in view of the now fairly well-established fact that glutathione is the coenzyme of the methylglyoxalase system, present in muscle as well as other tissues, which converts methylglyoxal into lactic acid.

It has been shown the iodoacetate destroys the glutathione in a muscle extract,²⁷ and thereby inhibits the glyoxalase. This would provide an excellent explanation of the effect of iodoacetate on intact muscles, if methylglyoxal were an essential intermediate step in the formation of lactic acid from glycogen; but Lohmann finds that although iodoacetate inhibits lactic acid production in his muscle extracts, it cannot be by reacting with glutathione since glycolysis of

glycogen occurs in muscle extracts rendered free of glutathione by dialysis. We must, from the facts available at present, suppose that there are two alternative routes leading to lactic acid: One of them involving methylglyoxal as an intermediate and both of them inhibited, though for different reasons, by iodoacetate. It was pointed out in the section on heart muscle that a frog heart exhausted in oxygen-free alkaline Ringer's fluid could be caused to recover by the addition of glucose to the medium. It is a point of noteworthy importance that the addition of methylglyoxal produces almost as good an effect.²²

To return to the glycolytic system studied by the schools of Meyerhof and Embden, it seems that the earlier stages of glycolysis consist

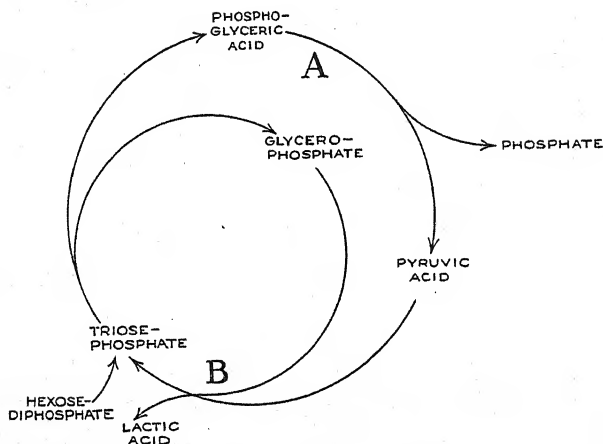


Fig. 50.—An attempt to depict graphically Meyerhof's view of the events leading to the production of lactic acid from hexosediphosphoric ester. Two molecules of triosephosphate (derived from hexosediphosphoric ester) are changed by the transference of hydrogen into one of phosphoglyceric acid and one of glycerophosphoric. The former loses phosphate and water, becoming pyruvic acid, which reacts with the molecule of glycerophosphate to give a molecule of lactic acid and regenerate a molecule of triosephosphate, which can enter the cycle again. There is evidence that fluoride interferes mainly at A, while iodoacetate appears to interfere at B.

in the formation in succession of glucose, hexosemonophosphoric ester, hexosediphosphoric ester; and from this point onward a cycle of events occurs,²⁸ which might be described as a chemical pulley wheel, such as is depicted in Fig. 50.²⁸

The Glycolytic Mechanism.—In studying the details of the glycolytic mechanism, it must not be forgotten that the lactic acid production is not the object of the mechanism, but appears rather to be a regrettable by-product. The mechanism exists, according to the present view, for the production of energy without the aid of oxygen; and there is evidence even in the cell-free muscle extracts as to the object, or one of the objects, for which this energy is wanted. This is the synthesis of adenosine triphosphate (adenylpyrophosphate),²⁹ which is

an endothermic reaction, and is found to occur only in alkaline muscle extracts in which glycolysis is occurring simultaneously. But even this is not an end in itself, for it is found that this ester breaks down again in the muscle extract simultaneously with the formation of creatine-phosphoric acid. In the absence of any glycolysis this phosphagen synthesis can be brought about by the addition to the muscle extract of adenosine triphosphate, and the phosphagen synthesis is found to be proportional to the extent of breakdown of the adenosine triphosphate.³⁰ This substance could therefore be regarded as a carrier of energy from the glycolytic system to the phosphagen-synthesizing system.

Non-irritable and Normal Muscles.—It is probably within the experience of everyone who has worked with isolated frog sartorii that occasionally a muscle, even though freshly dissected, will contract only feebly on stimulation, or fail to contract at all. It has been known empirically that this unfortunate aberration is less frequent if fairly liberal use is made of Ringer's solution during the dissection, and the conclusion has been drawn that the spontaneous inexcitability is due to dryness. The larger gastrocnemius muscle rarely shows this effect, and it can be induced in the skinned hind limbs only by keeping them for several days at 0° C. in oxygen.³¹ It is not easy to distinguish chemically non-irritable muscles from normal ones. Their contents of inorganic phosphate, phosphagen and lactic acid are normal. Kept in nitrogen they evolve heat at about the same rate as normal muscles, but produce lactic acid rather more slowly: Heat production per unit of lactic acid produced (the calorific quotient) proves on this account to be much higher than normal.³² The rate of heat production in oxygen is slightly more than double the rate in nitrogen as is the case with normal muscles. Treated in appropriate ways, such inexcitable muscles pass into rigor with the usual production of lactic acid; chopped up and suspended in alkaline buffer, the various phosphoric esters break up normally;³³ yet stimulation produces no contraction and no change in the lactic acid, total carbohydrate or phosphagen content. Their osmotic pressure as indicated by swelling in hypotonic salt solution is higher than normal,³¹ yet their electrical conductivity is little more than half that of a normal muscle.³⁴

That the spontaneous inexcitability of the sartorius muscle is not due to drying is shown by the fact that it sets in even in atmospheres saturated with water vapor, or if the muscle is kept under paraffin or mercury. Moreover, if the muscle is dissected with the use of Ringer's solution, or if soaked for twenty minutes or so after dissection, it will not become inexcitable when subsequently left in dry air, until the appearance of desiccation is very marked. The restorative effect of Ringer's solution is therefore not due to its water content.³⁵

It was found that if a small quantity of Ringer's fluid be used to restore excitability to a number of muscles in succession, it loses its curative properties and is then found to contain from three to four

times as much potassium as it contained initially.³⁶ Now it is an old observation that Ringer's solutions containing four times the normal potassium content will induce inexcitability in normal muscles, and if we couple this with the fact that the potassium of a normal muscle does not diffuse out readily into isotonic sugar solution (although the sodium can be lost rapidly and completely), we arrive at the explanation put forward by Dulière and Horton, that the shock of dissection together with the incidental small injuries results in leakage of potassium from the fibers where it is normally confined in fairly high concentration (about 0.4 per cent) into the surrounding plasma where it is normally present only in traces; and when the potassium has accumulated in the interspaces to a concentration about four times that characteristic of Ringer's fluid, inexcitability sets in. The shock or injury

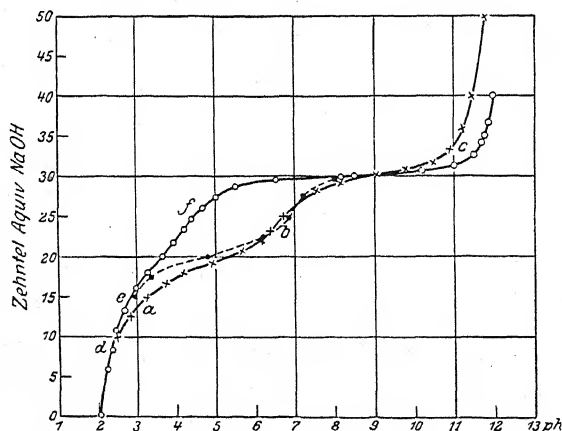


Fig. 51.—Titration curves of creatinephosphoric acid (—○—○—) and its hydrolysis products (—×—×—) together with the titration curve of an equimolecular mixture of creatine and phosphoric acid (—●—●—). (Taken from Meyerhof and Lohmann.⁴³)

of dissection is evidently only transient in character, since after soaking in Ringer's fluid the muscle does not again become inexcitable unless it is again injured as by pricking with a pin or by exposure to acid.³⁶ This view does not help us to understand the high osmotic pressure and low electrical conductivity of the inexcitable muscle; but it is possible that these are secondary effects, for the measurements in question were made on muscles taken from limbs kept several days at 0° C., whereas in the work on potassium diffusion use was made of simple sartorii which had become spontaneously inexcitable within a few minutes of dissection.

Hydrogen Ion Concentration.—A comparison of the titration curve of creatinephosphoric acid with the titration curves of its hydrolysis products³⁷ (Fig. 51) shows, as was first pointed out by Fiske and Sub-

barrow, that over the range pH 3 to pH 8 hydrolysis of creatinephosphoric acid results in a change in the reaction of the solution toward alkalinity. Now such a hydrolysis is the only reaction known to occur to any serious extent in the first few twitches of a previously resting muscle, and it becomes therefore a matter of great interest to decide whether such a muscle shows any tendency to become alkaline in the earlier stages of fatigue. It is of course well established that ultimately the tissue becomes markedly acid through the accumulation of lactic acid. By exercising isolated muscles in an atmosphere consisting of nitrogen with a small proportion of carbon dioxide, Meyerhof and Lipmann^{38, 39} were able to demonstrate in an elegant manner that there is indeed such a transient drift toward alkalinity (Fig. 52). For

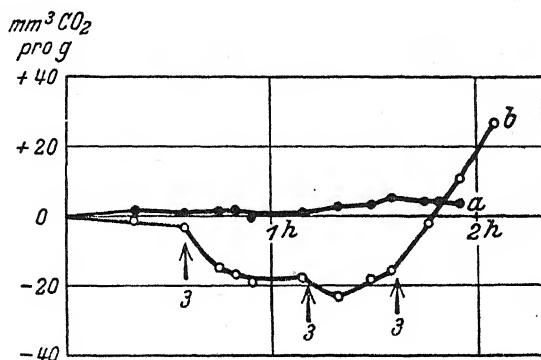


Fig. 52.—The CO_2 evolved from (+) or taken up by (—) frog muscles in an atmosphere of 95 per cent N_2 and 5 per cent CO_2 , over a period of two hours. (From Lipmann and Meyerhof.³⁸)

Curve a: Muscles resting throughout.

Curve b: Muscles given three bouts of activity (marked by arrows).

the muscles were found to absorb carbon dioxide from the gas mixture after each of the first few bouts of activity, though ultimately, as was to be expected, they expelled carbon dioxide as lactic acid accumulated within them. The effect was very marked if the gas mixture contained a high proportion of carbon dioxide, thereby inducing initially a somewhat acid reaction in the muscles. This was not surprising, for it had been demonstrated that the effect of an acid reaction in muscle extracts is to favor the enzyme system responsible for the breakdown of phosphagen and to inhibit the glycolytic ferment system. At a pH of 8 the phosphagen-hydrolyzing enzyme is almost inactive, and this fact may perhaps be related to the observation that muscles kept in a gas mixture containing very little carbon dioxide (about 0.3 per cent) showed very little absorption of carbon dioxide during the earlier stages of activity.

REFERENCES

1. Wolff, W. A., and Wilson, D. W.: *J. Biol. Chem.*, **95**, 495 (1932).
2. Broude, L.: *Z. Physiol. Chem.*, **217**, 56 (1933).
3. Clifford, W. M.: *Biochem. J.*, **15**, 725 (1921).
4. Meyerhof, A.: *Arch. di Sci. Biol.*, **12**, 536 (1928).
5. Eggleton, P., and Eggleton, G. P.: *Quart. Exp. Physiol.*, **23**, 391 (1933).
6. Eggleton, P.: *J. Physiol.*, **70**, 294 (1930).
7. Hill, A. V., and Kupalov, P. S.: *Proc. Roy. Soc. (London)*, **106 B**, 445 (1930).
8. Devadatta, C. S.: *J. Physiol.*, **79**, 194 (1933).
9. Nachmannsohn, D.: *Biochem. Z.*, **208**, 237 (1929).
10. Meyerhof, O., and Schulz, W.: *Biochem. Z.*, **236**, 54 (1931).
11. Ritchie, A. D.: *J. Physiol.*, **78**, 322 (1933).
12. Lundsgaard, E.: *Biochem. Z.*, **217**, 162 (1930).
13. Hill, A. V., and Parkinson, J. L.: *Proc. Roy. Soc. (London)*, **108 B**, 148 (1931).
14. Hartree, W.: *J. Physiol.*, **79**, 492 (1933).
15. Lehnartz, E.: *Klin. Wochschr.*, **10**, 27 (1931).
16. Meyerhof, O.: *Klin. Wochschr.*, **10**, 214 (1931).
17. Lundsgaard, E.: *Biochem. Z.*, **233**, 322 (1931).
18. Cori, G. T., and Cori, C. F.: *J. Biol. Chem.*, **99**, 493 (1933).
19. Fenn, W. O.: *J. Cell. Comp. Physiol.*, **2**, 233 (1932).
20. Clark, A. J., Gaddie, R., and Stewart, C. P.: *J. Physiol.*, **72**, 443 (1931).
21. Clark, A. J., Gaddie, R., and Stewart, C. P.: *J. Physiol.*, **75**, 311 (1932).
22. Stewart, C. P., and Gaddie, R.: *J. Physiol.* (In press.)
23. Clark, A. J., Eggleton, M. G., and Eggleton, P.: *J. Physiol.*, **75**, 332 (1932).
24. Meyer, K.: *Biochem. Z.*, **193**, 139 (1928).
25. Meyerhof, O., Lohmann, K., and Meyer, K.: *Biochem. Z.*, **237**, 437 (1931).
26. Lohmann, K.: *Biochem. Z.*, **262**, 152 (1933).
27. Dickens, F.: *Nature*, **131**, 130 (1933).
28. Meyerhof, O.: *Nature*, **132**, 357 (1933).
29. Lehnartz, E.: *Klin. Wochschr.*, **7**, 1225 (1928).
30. Meyerhof, O., and Lohmann, K.: *Naturwissenschaften*, **19**, 575 (1931).
31. Foster, D. L., and Moyle, D. M.: *Biochem. J.*, **15**, 334 (1921).
32. McCullagh, D. R., and Case, E. M.: *Biochem. J.*, **25**, 1220 (1931).
33. Edsall, J. T.: *Biochem. J.*, **20**, 569 (1926).
34. Thomson, D. L.: *J. Physiol.*, **65**, 214 (1928).
35. Dulière, W., and Horton, H. V.: *J. Physiol.*, **67**, 152 (1929).
36. Horton, H. V.: *J. Physiol.*, **70**, 389 (1929).
37. Fiske, C. H., and Subbarow, Y.: *J. Biol. Chem.*, **81**, 629 (1929).
38. Lipmann, F., and Meyerhof, O.: *Biochem. Z.*, **227**, 84 (1930).
39. Meyerhof, O., Möhle, W., and Schulz, W.: *Biochem. Z.*, **246**, 285 (1932).
40. Meyerhof, O., and Nachmannsohn, D.: *Biochem. Z.*, **222**, 1 (1930).
41. Eggleton, P., and Eggleton, G. P.: *J. Physiol.*, **68**, 193 (1929).
42. Nachmannsohn, D.: *Biochem. Z.*, **196**, 73 (1928).
43. Meyerhof, O., and Lohmann, K.: *Biochem. Z.*, **196**, 49 (1928).

CHAPTER XXVII

THE CHEMISTRY OF THE INTEGUMENT

THE skin and its accessory structures are of the greatest importance to the organism owing to their protective capacity. An unbroken skin is the most adequate defense against harmful environmental factors, such as bacterial toxins and the introduction of micro-organisms themselves. The heat regulation is made largely through the agency of the skin. The incidence of rays inimical to the delicate structures of the body is mitigated by the skin pigments and other chemical constituents lying within the integument. Finally, trauma is rendered less effective through the defense features of the cuticle, hair, nails, and corium. It is interesting to examine the skin constituents to determine how they have been adapted to the part they play in defense.

The Morphology of the Skin.—The skin is derived from the ectoderm, the outermost of the three primary layers of the embryo. Aside from some of the lower animals of the vertebrate series, like the lancelet, or *Amphioxus*, the skin is composed of two characteristic layers, the epidermis and the corium. Underlying the corium is the musculature from which the two skin layers may be separated mechanically or by the aid of reagents or heat. The skin, then, is the integument lying on the outside of the skeletal muscles.

The epidermis consists of many layers of cells which are constantly being formed from within (that is, the zone nearest the corium) and as they become pushed outward, become flat and eventually die. They then form the "horny" layer of the skin which is being eroded constantly. In places where pressure is exerted more or less continuously, as in the heel and palm, the epidermis is thick and the cuticle forms a zone of keratin-walled cells which renders the inner parts of the heel safe from all save heavy bruises.

The zone of more active cell proliferation nearest the corium was named for the famous Italian biologist, Malpighi, but it is also known as the *stratum germinativum* and also as the *stratum mucosum*. There is a less clear zone, the *stratum lucidum*, between the malpighian layer proper and the corium lying beneath.

The corium consists largely of white fibrous tissue composed of collagen. Distributed among the fibers of this tissue are blood vessels and interstitial cells which are removed when this layer is used for leather by the process of "bating," an enzyme procedure.

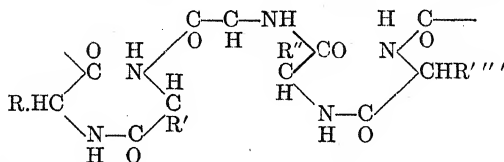
Integumentary glands arise from the epidermis but dip down into the corium. Hair follicles also penetrate into the corium from the upper layer. Elastic fibers, elastin chemically, intertwine among the

follicles, perhaps acting as a hold-fast. Other integumentary structures are the scales and teeth that develop within the malpighian layer and protrude upward, generally being provided with a protective covering, either bony or keratogenous.

The weight of skin in a human adult is approximately 18 per cent of the body weight¹ and the skin surface approximates 1.5 square meters.²

Among the chemical constituents of the integument are keratin, collagen, elastin and reticulin.

Keratin.—This substance is insoluble in ordinary reagents in the cold, but is affected by alkali, especially at elevated temperatures. The intimate structure of wool, which is a typical keratogenous structure, has been attacked recently both from the physical as well as from the chemical point of view.³ The keratin-bearing structures are minute micelles about 200Å (200 × 10⁻⁸ cm.) wide and 1000Å long, arranged with their long axes parallel to that of the fiber of wool. Chemically, the micelle consists of long peptide chains crinkled into ringlike structures, requisite to explain the refractory properties of keratin.⁴



the radicals R', R'', etc., being side chains of amino acids. In addition to the primary valences, these chains are bridged by covalences. Of the primary valences, that of the dipeptide, cystine, is the most important.⁵

The amino acid content of keratin has been studied by Vickery⁶ and others⁷ by modern methods of analysis and the general conclusion is that there is not any great variation in the number or amounts of amino acids in the various samples.

Abderhalden has attempted to determine the composition of ox-horn by stepwise hydrolysis.²³ Previously he had done this for silk and found the dipeptide, alanyl-glycine; and later still he isolated glycyl-alanine, glycyl-tyrosine, alanyl-glycyl-tyrosine and glycyl-alanyl-glycyl-tyrosine.^{24, 25} Finally, from silk, a pentapeptide, glycyl-seryl-prolyl-tyrosyl-proline and a tetrapeptide, seryl-prolyl-tyrosyl-proline were isolated.²³

The three amino acids of keratin, glutamic acid, cystine and glycine may represent a molecule of a precursor, glutathione, which seems to accumulate as the epidermis is formed and to disappear as keratin is formed.²⁶

The cystine of keratin is reported to act in oxidation-reduction in a manner similar to glutathione.²⁷

THE AMINO ACID CONTENT OF KERATIN DERIVED FROM DIFFERENT SOURCES

| Amino acid | Source | Authority (see references) | Per cent |
|---------------------|----------------|-------------------------------|----------|
| Glycine..... | Ox-horn | 8 | .30 |
| | Sheep-horn | 9 | .40 |
| | Goose feathers | 10 | 2.60 |
| | Horsehair | 11 | 4.70 |
| | Silk | 12 | 40.50 |
| | Wool | 9 | .58 |
| | Skin of python | 13 | .00 |
| Alanine..... | Ox-horn | 8 | 1.20 |
| | Sheep-horn | 9 | 1.60 |
| | Goose feathers | 10 | 1.80 |
| | Horsehair | 11 | 4.70 |
| | Wool | 9 | 4.40 |
| | Silk | 12 | 25.00 |
| Valine..... | Ox-horn | 8 | 1.20 |
| | Sheep-horn | 9 | 4.50 |
| | Goose feathers | 10 | .50 |
| | Horsehair | 11 | .90 |
| | Wool | 9 | 4.50 |
| | Silk | 12 | .00 |
| Leucine..... | Ox-horn | 8 | 18.30 |
| | Sheep-horn | 9 | 15.30 |
| | Goose feathers | 10 | 8.00 |
| | Horsehair | 11 | 7.10 |
| | Wool | 9 | 11.50 |
| | Silk | 12 | 2.50 |
| Proline..... | Ox-horn | 8 | 3.60 |
| | Sheep-horn | 9 | 3.70 |
| | Goose feathers | 10 | 3.50 |
| | Horsehair | 11 | 3.40 |
| | Wool | 9 | 4.40 |
| | Silk | 12 | 1.00 |
| Hydroxyproline..... | Silk | 12 | .00 |
| Serine..... | Ox-horn | 8 | .70 |
| | Sheep-horn | 9 | 1.10 |
| | Goose feathers | 10 | .40 |
| | Horsehair | 11 | .60 |
| | Wool | 9 | .10 |
| | Silk | 12 | 1.80 |
| Phenylalanine.. | Sheep-horn | 9 | 1.00 |
| | Silk | 12 | 1.50 |
| Aspartic acid..... | Ox-horn | 8 | 2.50 |
| | Sheep-horn | 9 | 2.50 |
| | Goose feathers | 10 | 1.10 |
| | Horsehair | 11 | .30 |
| | Wool | 9 | 2.30 |
| | Silk | 12 | .00 |
| Glutamic acid..... | Ox-horn | 8 | 3.00 |
| | Sheep-horn | 9 | 17.20 |
| | Goose feathers | 10 | 2.30 |
| | Horsehair | 11 | 3.70 |
| | Wool | 9 | 12.90 |
| | Silk | 12 | .00 |
| | Ox-hoof | 14 | 18.00* |
| | Cow-hoof | 14 | 13.80* |
| | Horse-hoof | 14 | 18.10* |

| Amino acid | Source | Authority (see references) | Per cent |
|---------------------|---------------------|-------------------------------|----------|
| Tyrosine..... | Ox-horn | 8 | 4.60 |
| | Sheep-horn | 9 | 3.60 |
| | Goose feathers | 10 | 3.60 |
| | Horsehair | 11 | 3.20 |
| | Wool | 9 | 2.90 |
| | Silk | 12 | 11.00 |
| Cystine..... | Sheep-horn | 9 | 7.50 |
| | Wool | 9 | 7.30 |
| | Silk | 12 | 11.00 |
| | Human hair | 15 | 14.03 |
| | Human nails | 15 | 5.15 |
| | Horsehair | 15 | 7.98 |
| | Horse-hoof | 15 | 3.20 |
| | Ox-hair | 15 | 7.27 |
| | Ox-hoof | 15 | 5.37 |
| | Hog bristles | 15 | 7.22 |
| | Hog-hoof | 15 | 2.17 |
| | Python skin | 13 | .14 |
| | Python skin | 16 | 5.30 |
| | Human hair | 6 | 16.50 |
| | Wool | 6 | 10.00 |
| | Goose feathers | 16 | 6.40 |
| | Silk | 16 | .00 |
| Arginine..... | Sheep-horn | 9 | 2.70 |
| | Python skin | 13 | 1.35 |
| | Silk | 12 | .00 |
| | Ox-horn | 17 | 4.68 |
| | Cow-hair | 18 | 4.50 |
| | Cow-horn | 9 | 2.70 |
| | Turtle keratin | 19 | 3.40 |
| | Sheep-wool | 20 | 10.20 |
| | Sheep-wool | 21 | 7.80 |
| | Human hair | 6 | 8.00 |
| | Horsehair | 6 | 7.60 |
| | Coral (Gorgonia) | 16 | 7.30 |
| | Coral (Plexaurella) | 16 | 6.60 |
| | Snake skin | 16 | 6.10 |
| | Goose feathers | 16 | 5.00 |
| | Silk | 16 | .74 |
| Lysine..... | Sheep-horn | 9 | .02 |
| | Silk | 12 | 1.50 |
| | Sheep-wool | 20 | 2.80 |
| | Sheep-wool | 21 | 2.30 |
| | Human hair | 6 | 2.50 |
| | Snake skin | 16 | 1.40 |
| | Goose feathers | 16 | 1.04 |
| | Coral (Gorgonia) | 16 | 2.75 |
| | Coral (Plexaurella) | 16 | 3.20 |
| | Silk | 16 | .25 |
| Histidine..... | Silk | 12 | .75 |
| | Silk | 16 | .07 |
| | Human hair | 6 | .50 |
| | Sheep-wool | 21 | .66 |
| | Sheep-wool | 20 | 6.90 |
| Norvaline..... | Cow-horn | 22 | 2.00 |
| Hydroxyproline..... | Cow-horn | 22 | .50 |

* As the hydrochloride.

Usually, there is much interest in the question concerning the chemical differences in human hair of different colors. We present a table derived from Rutherford and Hawk:²⁸

| Hair | S, per cent | N, per cent | C, per cent | H, per cent | O, per cent |
|----------------------|----------------|----------------|----------------|----------------|----------------|
| Indian..... | 4.8 | 15.4 | 44.0 | 6.5 | 29.1 |
| Negro..... | 4.8 | 14.9 | 43.8 | 6.3 | 30.4 |
| White (aged)..... | 5.0 | 15.2 | 44.6 | 6.3 | 28.8 |
| White adult..... | 5.2 | 15.1 | 44.9 | 6.4 | 28.6 |
| White, red hair..... | 6.07 | 15.1 | 45.2 | 6.6 | 26.9 |
| White child..... | 4.9 | 14.5 | 43.2 | 6.4 | 30.8 |

The differences are small. What the significance is that red hair of the Caucasian contains more sulfur than the hair of other color, or that of other races, is unknown. To the geneticist, red hair is "diluted" black; that is, the pigment is not as thickly distributed in the hair.

The isoelectric point of keratin is given as 4.8.²⁸ The range of stability for wool is stated to be from pH 4-7. In stronger alkali, cystine is attacked; as much as 66 per cent of the total sulfur of keratin is removable from cystine.²⁷ Keratin swells below pH 1 and above pH 11 in distilled water, a marked increase in volume being found from pH 12-13.²⁹ At this pH, alkali causes not only swelling but peptization. Hydrosulfite causes a similar effect.

Roentgen ray analysis gives three pictures of keratins.³⁰ Silk seems to be under stress, for its picture resembles hair keratin under a stretching force.

The molecular condition of keratin may be put in terms of size of particles penetrating the fiber: intermicellar spaces admit substances of the general molecular magnitude of propyl alcohol,³ but by pretreatment of the fiber with agents of larger molecular size mixed with water, large molecular substances, as large as octyl alcohol, may be introduced. When fibers are completely free from water, their roentgen ray picture is altered. Liquids that accumulate in the intermicellar spaces serve to cause an extension of the fiber by straightening out the coiled peptide chains. The reason for this is that we introduce a higher dielectric when we dry the fiber and, *vice versa*, decrease the dielectric force by adding water.

The deposition of keratin is connected with the amount of pressure exerted upon the epidermis. Thus Dahlgren was able experimentally to modify the thickness of the keratin layer by varying the pressure upon the structures (personal communication).

The contrast between keratin, on the one hand, and collagen, on the other, relates in part to the presence in the keratin of cystine and tyrosine in liberal amounts and to their absence or slight concentration in collagen, also to the small content of hydroxyproline in keratin and its high content in collagen.

Collagen.—Collagen is found in the white fibrous tissue beneath the epidermis.^{31, 32} The tissue is in bundles separated by a sort of sarcolemma. Interstitial cells, blood vessels and lymph spaces separate the bundles from one another. The size and shape of the bundles varies with the location. In the skin, the bundles are short and thick and cause the "pebbling" of the surface of the skin; while in tendons, the bundles are long and attenuated. In animals like reptiles which shed their skin more or less intact, a waxy layer forms between the epidermis and the cuticle, and the cuticle is shed. No collagen, then, or other structure is concerned in this ecdysis and the shed "skin" is not truly skin, which includes the corium and epidermis.

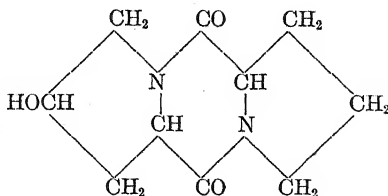
The intimate structure of the collagen bundle is not clear. Probably, it involves a fibrous structure, the fibers being bound into bundles by means of "hulls,"³² connective-tissue cells something like those that surround the nerve fibers. When collagen is converted into gelatin these "hulls" are torn and the fibers are free. The fibers are composed of micelles,³³ each being about $10m\mu$ (10×10^{-10} cm.). The lattice spaces are 20\AA (20×10^{-8} cm.), being the coefficient of absorption.³⁴ Sex differences in collagen are recorded,³⁵ the male collagen being more "acid-producing," having greater ability to fix reagents like those used in tanning and showing less iodine fixing capacity, less swelling capacity; the specific viscosity is higher, the specific rotation is lower and the male collagen is richer in basic amino acids.

The chemical composition of collagen is rather the chemistry of gelatin which is produced from it when collagen is boiled. The analyses of Dakin³⁶ are the most complete. In addition we give data from other sources:

| Amino acid | Per cent | Amino acid | Per cent |
|--------------------|----------|---------------------|-------------------|
| Glycine..... | 25.5 | Tyrosine..... | .00 |
| Alanine..... | 8.7 | Proline..... | 9.5 |
| Valine..... | .0 | Hydroxyproline..... | 14.10 |
| Leucine..... | 7.1 | Tryptophan..... | .00 |
| Serine..... | .4 | Histidine..... | .9 |
| Aspartic acid..... | 3.4 | Arginine..... | 8.2 |
| Glutamic acid..... | 5.8 | Lysine..... | 5.9 |
| Cystine..... | .0 | Methionine..... | .97 ³⁷ |
| Phenylalanine..... | 1.4 | | |

Concerning tyrosine, Dakin says: "It is a safe deduction that tyrosine is not an integral part of the gelatin molecule."³⁶ Siegfried, using intestinal mucosa, suggested that tyrosine is derived from the reticulín that accompanies collagen in such tissues.³⁸ The writer detected minute traces of tyrosine in purified collagen.³⁹ In

addition to the substances listed above, Van Slyke and Hiller found in gelatin a basic constituent that remains unidentified, although it may be a dipeptide, dihydroxyproline-alanine.^{40, 41} Dakin³⁶ encountered hydroxyprolin-anhydride:



In 1924, Abderhalden⁴¹ suggested two factors in molecular weights of large molecule proteins, namely, simple unit weight and multiple or molecular aggregate weights. The following table gives determinations made by several observers:

| Observer | Aggregate Mol. Wt. |
|--|--------------------|
| Barger and Rast ⁴² | 19,000 |
| Biltz ⁴¹ | 30,000 |
| Krishnamurti and Svedberg ⁴ | 11,000 |
| Lloyd ⁵ | 10,000 |

The simple molecular weight of gelatin may be inferred by analogy with determinations of molecular weights of other proteins, such as lactalbumin which has an aggregate molecular weight of about 12,000 but a simple molecular weight of about 1000.⁴⁶

The isoelectric point of collagen has been much investigated. Hitchcock found it to be at pH 4.7.^{47, 48, 49}

Collagen is digested by pepsin-hydrochloric acid to polypeptides,⁵⁰ but it is affected by trypsin only at temperatures above 40° C.⁵¹ The tissue "autolytic" enzymes do not hydrolyze collagen at body temperature.⁵⁵

While special protein-split products have been described for trypsin-digested collagen,⁵² it is improbable that any variation from the usual products of protease action is obtained. Abderhalden determined that the bonds affected by trypsin are different from those that are involved in methylation or benzylation.⁵³ Trypsin seems to attack, first, the hulls binding the fibers, then later the fibers themselves.⁵⁴

Gelatin is produced when collagen is boiled for a time in distilled water. The time necessary to convert collagen into gelatin varies with the kind of collagen; skin collagen requires about six hours while collagen derived from *tendo achillis* is obtained more quickly. The prevalent view seems to be that conversion of collagen to gelatin involves only a physical change.³⁴ There is no increase in amino nitrogen as one would expect if a chemical change, like hydrolysis, were involved.⁵⁶ x-Ray diagrams of air-dried collagen and air-dried gelatin are identical ($d = 4.5\text{\AA}$).⁵⁷

Elastin.—Elastin is a protein that permeates the region around the

roots of the hairs and glands.³¹ Unlike collagen, it is not bound into fibers by hulls, but its fibers are threadlike, running about through the tissues and giving the appearance of wires loosely wound around the hair roots. What function elastin performs is unknown; but one gets the impression that it serves to regulate the tension in the parts of the skin in which it occurs. It may aid in fixing a "fulcrum" for the action of the *erector pilae* or small muscles whose contraction erects the hairs, and, also, in the compression of the glands. The chemistry of elastin has yet to be investigated.

Reticulin.—Skin reticulin is another substance that needs careful study. It has been found associated with collagen in other parts of the body.³⁸

The Mineral Chemistry of the Skin.—Brown summarizes the earlier work on the mineral content of the skin of different animals, and adds his own work:⁶²

| Animal | Ca (in mg.) | Mg (in mg.) | Na (in mg.) | K (in mg.) |
|-------------|-------------------|-------------------|-------------------|------------------|
| Man..... | 34-59 | 20-38 | 298-408 | 168-339 |
| Dog..... | 31-58 | 21-37 | 155-250 | 158-395 |
| Rabbit..... | 51-86 | 17-52 | 116-243 | 102-188 |

Loewy and Cronheim have given similar figures for the skins of the rat and guinea-pig:⁶⁶

| | Rat (in mg.) | Guinea-pig (in mg.) |
|---------|-----------------|------------------------|
| Cl..... | 633-1441 | 507-1817 |
| K..... | 371-446 | 215-244 |
| Ca..... | 63.7-86.6 | 100 |
| Na..... | 212-247 | 252-411 |
| Mg..... | 20.3-27.5 | 30.3-54.5 |

The water contents of the skin of rat and guinea-pig are:

| | Per cent |
|-----------------|-------------------------|
| Rat..... | 58.0-68.8 |
| Guinea-pig..... | 60.6-72.7 ⁶⁶ |

These figures agree with the water content of skins and hides used in the making of leather.⁶⁸

The fat content of the skin is made up of neutral fat, cholesterol, cholesterol esters and lipid substances.

| Source | Kind of lipid | Amount | Reference |
|--------------------------------------|-------------------|-----------|-----------|
| Rat..... | Total lipid | 6.41-17.8 | (66) |
| Guinea-pig..... | Total lipid | 1.28-4.26 | (66) |
| Human (female)..... | Total lipid | 7.14 | (69) |
| Human (both sexes) (epidermis)..... | Phospholipid | 0.62 | (70) |
| Human (epidermis, basal)..... | Phospholipid | 2.62 | (70) |
| Human (epidermis, Str. corneum)..... | Phospholipid | 0.14 | (70) |
| Human (epidermis)..... | Cholesterol, free | 0.90 | (70) |
| Human (basal layer)..... | Cholesterol, free | 1.56 | (70) |
| Human (Str. corneum)..... | Cholesterol, free | 0.73 | (70) |
| Human (epidermis)..... | Fatty acids | 1.71 | (70) |
| Human (basal layers)..... | Fatty acids | 3.94 | (70) |
| Human (Str. corneum)..... | Fatty acids | 2.87 | (70) |

Klose⁷¹ finds skin-fat to be 0.1—50 per cent of the total weight of the dry skin;⁷¹ 87 per cent of the cholesterol in the integument is free.⁷² The phospholipid content of human skin is 2.5—3.1 per cent of the total lipid.⁷³ Ergosterol has been found in skin to the extent of about $\frac{1}{600}$ of the total sterol.⁷⁴

Sugar occurs in skin in two forms, as free and as bound sugar. Free sugar is fifteen times less than bound sugar. Bound sugar is twice as high as the bound sugar of blood. Bound sugar increases with the increase in carbohydrate intake. Folin⁷⁶ finds "true" or fermentable sugar in skin to be 56 mg. per 100 Gm., or about 62 per cent of blood sugar. Apparently, this sugar is readily removed by washing.⁵⁵

Permeability of the Skin to Irradiation.—Taking the "coefficient of absorption" as the expression:

$$\frac{J}{J_0} = \frac{t'}{t}$$

where J is intensity of transmitted light, J_0 that of incident light, t' and t the times required to produce an image of the same intensity by transmitted and incident light, respectively, Hasselbalch found for the whole skin of a human being the following:⁶³

| | | |
|-------------------------------|-------|------|
| λ (in $\mu\mu$)..... | 437.0 | 3.13 |
| Coefficient..... | 2.3 | 5.2 |

For the epidermis:

| | | | | | | | |
|------------------|-----|-----|-----|-----|------|------|-----|
| λ | 404 | 360 | 334 | 313 | 302 | 294 | 289 |
| Coefficient..... | 2.3 | 4.2 | 6.6 | 8.5 | 12.4 | 17.5 | 39 |

Lucas finds much higher absorption, the variation being from 1.5 to 30 times Hasselbalch's figures.⁶⁴ The curves of absorption are similar to those of absorption with tryptophan, cystine and tyrosine, as well as the proteins serum-albumin, and casein. Cystine has practically no opacity for ultra-violet light in the "curative" zone (294 to 313), but tyrosine and tryptophan exert a retarding effect.

Skin pigments are discussed by Dejust.⁶⁵ The pigments are of the nature of melanins.

The term *exoskeleton* is applied to structures secreted by the integument, such as scales, hair, feathers, hoofs, nails and horns. The manner in which such parts are formed is more or less similar. The chief constituent is keratin, supported by bone or lime salts. In true horns, which are branched only rarely (*e. g.*, the antelope), the keratin overlies a bony framework. In antlers there is a bony core lying within the fibrous and collagenous layers, on the outside of which lies the epidermis.⁷⁹ The layers external to the bone constitute the "velvet" which is rubbed off at maturity. Unlike true horn, then, the antler at maturity is wholly bone.

A similar relation exists in the large scales of certain fishes, where,

in the developmental stages, keratin covers the bone proper, but may wear away, exposing the bone. Hair, feathers and hoofs are formed in pits in the skin. Hair is held very lightly. The tanner removes hair by means of lime, sulfide, amines or bacterial action, or by means of tryptic enzymes. Apparently, the delicate attachment of the hair to the skin through the bulb of the hair is destroyed, and the hair, along with the epidermis lining the hair follicle, is easily removed by a knife in the process of depilation or "scudding."

Hair grows discontinuously, some follicles being active while others rest, as in the case of renal glomeruli. Races differ in hirsuteness, the Mongolian and Oriental races (like the Chinese) have sparse hairiness; but it is not because of inability to grow hair, for adrenal tumors cause abundant hair development in Chinese.⁸¹ The female of all races shows hairiness on the face and body at menopause and especially after ovariectomy or lesions of the hypothalamus. Exoskeletal substances, like hair, kept free of oxygen in development, remain colorless, but on admitting oxygen, pigmentation occurs.⁸²

Cystine is so characteristic of hair that its significance may be postulated, and through the observations of Beadles, Braman and Mitchell, it appears that cystine added to a diet induces additional hair growth.⁸³ In this case a low protein diet was used to insure that the growth was not due simply to increased metabolism through a plethora of protein food. The cutting of hair seems to accelerate its growth.⁸⁴

The skin secretes certain substances such as oils and waxes, the purpose of which is not merely to lubricate the skin, but also to provide heat.⁸⁵

Sweat.—Two sets of glands occur in the skin: those connected with the hair follicles and those having pores independently. The former secrete an oil, the latter the perspiration. While the first kind occur typically in connection with hairs, they may be present where the actual rachis of the hair is absent, as in the prepuce and labia.

A distinction is made between "eccrine" and "apocrine" perspiration. Eccrine perspiration is that which is secreted throughout the skin, while apocrine sweat is secreted in limited areas, such as the axilla, groin and pudenda. The reaction of apocrine sweat is alkaline,⁸⁶ eccrine sweat is acid (pH 4.5),⁸⁷ the acidity being due to lactic acid.^{87, 88} This organic acid plays an important rôle in conserving bases from the blood that otherwise would be excreted through the skin. It has been suggested, too, that lactic acid may accelerate the activity of the sweat glands.

All substances found in blood plasma, save protein and carbonate, occur in sweat. Protein does not penetrate the tissues and the acid reaction prevents the carbonates from becoming effective. The carbonates are thus saved for the alkali reserve.⁸⁹

Composition of Sweat⁸⁷
(eccrine) (as 0.1 N)

| | Na | K | Ca | Cl | Lactic acid | pH |
|----------------------|--------|-------|-----|----|-------------|-----|
| High water loss..... | 82 cc. | 5.1 | 5.7 | 85 | 25.1 | 4.1 |
| Low water loss..... | 81 cc. | 10.05 | 5.3 | 69 | 34.0 | 4.3 |

The sodium chloride content is given by Talbert as 55-65 mg./100 cc.⁹³

The buffer value of perspiration is equivalent to 0.02 normal acid.

The total ash during work is about 0.5 Gm.⁹⁴

The content of lactic acid is given higher values by some (82.05 to 160.4 mg. per 100 cc.).⁹⁰

Uric acid is reported as about 0.017 mg. per 100 cc.;⁹⁰ urea, 0.16-0.28 mg. per 100 cc.;⁹¹ ammonia, 5-35 mg. per 100 cc.⁹² The amino acid content of perspiration is 1.57-4.76 mg. per 100 cc. The sugar (fermentable) content is given as 5.6-40 mg. per 100 cc.⁹⁶ The water loss in excessive exercise may be as high as 1 liter per hour.⁹⁷

The fat of sweat is a mixture of many kinds of lipids.

Tears are watery secretions and are alkaline in reaction. Water makes up 99 per cent; protein (soluble and that in epithelial cells found in tears), 0.22 per cent; fat, 0.30 per cent; NaCl, 0.42 per cent; salts other than NaCl (phosphates, etc.), 1.5 per cent.

Vernix caseosa is a waxlike substance covering the fetus at birth. From various sources, we know that the chemical composition is somewhat as follows:

| | Per cent |
|-------------------------|----------|
| Water..... | 70 |
| Fatty substances..... | 11 |
| Nitrogenous matter..... | 19 |
| Ash..... | 6 |

Smegma is a term applied to the secretions of the prepuce and corresponding parts of the female. The chemical composition is:

| | Per cent |
|---------------------------------|----------|
| Water-extractable material..... | 6.1 |
| Fat-soluble (ether)..... | 52.8 |
| Fat-soluble (alcohol)..... | 7.4 |
| Protein material..... | 5.6 |
| Salts..... | 9.7 |
| Insoluble residue..... | 18.4 |

WITHROW MORSE.

REFERENCES

1. Vierordt, H.: *Anatomische, physiologische und physikalische Daten und Tabellen* (1893).
2. Morse, W.: *Applied Biochemistry* (1927).
3. Speakman, J. B.: *Proc. Roy. Soc. (London)*, **132A**, (1931).
4. Astbury, R. S., and Street, W. C.: *Philos. Tr.*, **230A** 75 (1931).
5. Astbury, R. S., and Woods, M. F.: *J. Textile Inst.*, **23T**, 17 (1932).
6. Vickery, H. B., etc.: *J. Biol. Chem.*, **86**, 107 (1930).
7. Mullin, C. E., and Hunter, H. L.: *Textile Colorist*, **53**, 599 (1931).
8. Fischer, E., und Doerpinaus, T.: *Z. physiol. Chem.*, **36**, 462 (1902).
9. Abderhalden, E., und Voitinovici, A.: *Z. physiol. Chem.*, **52**, 348 (1907).
10. Abderhalden, E., und LeCount, E. R.: *Z. physiol. Chem.*, **46**, 31 (1905).

11. Abderhalden, E., und Wells, H. G.: *Z. physiol. Chem.*, **46**, 31 (1905).
12. See 11.
13. Oikawa, S.: *J. Biochem. (Jap.)*, **5**, 57 (1925).
14. Abderhalden, E., und Fuchs, D.: *Z. physiol. Chem.*, **57**, 339 (1908).
15. Buchtala, H.: *Z. physiol. Chem.*, **52**, 474 (1907).
16. Block, R. J., and Vickery, H. B.: *J. Biol. Chem.*, **93**, 113 (1931).
17. Fuert, O. von, und Deutschberger, O.: *Biochem. Z.*, **186**, 139 (1927).
18. Argyris, A.: *Z. physiol. Chem.*, **54**, 86 (1907).
19. Keil, W.: *Ber.*, **59**, 202 (1926).
20. Marston, H. R.: *Australian Council of Science and Industrial Research, Bull. No. 38* (1928).
21. Vickery, H. B., and Block, R. J.: *J. Biol. Chem.*, **86**, 107 (1930).
22. Abderhalden, E., und Heyns, K.: *Z. physiol. Chem.*, **206**, 137 (1932).
23. Abderhalden, E., und Bahn, A.: *Z. physiol. Chem.*, **210**, 246 (1932).
24. Abderhalden, E.: *Z. physiol. Chem.*, **120**, 207 (1922).
25. Abderhalden, E., und Heyns, K.: *Z. physiol. Chem.*, **202**, 37 (1931).
26. Girond, A., et Buillard, H.: *Comt. rend. Soc. biol.*, **98**, 500 (1928).
27. King, A. T.: *J. Textile Ind.*, **18T**, 36 (1927).
28. Speakman, J. B., and Hirst, M. C.: *Nature*, **127**, 665 (1931).
29. Menschel, H.: *Arch. f. exp. Path. u. Pharmak.*, **110**, 1 (1925).
30. Marwick, T. C.: *J. Textile Sci.*, **4**, 31 (1931).
31. Turley, H. G.: *J. Am. Leather Chem. Assoc.*, **21**, 117 (1920).
32. Kaye, M., and Lloyd, D. J.: *Proc. Roy. Soc. (London)*, **96B**, (1924).
33. Bachmann, W.: *Z. anorg. Chem.*, **73**, 125 (1912).
34. Herzog, R. O., und Gonell, H. W.: *Ber.*, **58**, 2228 (1925).
35. Tadokoro, T., and Yoshimura, K.: *J. Agr. Chem. Soc.*, **7**, 52 (1931) (Japanese).
36. Dakin, H. D.: *J. Biol. Chem.*, **44**, 524 (1920).
37. Baernstein, H. D.: *J. Biol. Chem.*, **89**, 129; **97**, 663 (1932).
38. Siegfried, M.: *J. Physiol.*, **28**, 319 (1893).
39. Morse, W.: *J. Biol. Chem.*, **97** (Proc.), xxx (1932).
40. Sadikof, W. S.: *Biochem. Z.*, **179**, 326 (1926).
41. Abderhalden, E.: *Naturwissenschaften*, **12**, 719 (1924).
42. Barger, G., and Rast, K.: *J. Chem. Soc.*, **85**, 286 (1904).
43. Biltz, W.: *Z. physik. Chem.*, **91**, 719 (1916).
44. Krishnamurti, K., and Svedberg, T.: *J. Am. Chem. Soc.*, **52**, 2897 (1930).
45. Lloyd, D. J.: *Biochem. J.*, **14**, 147 (1920).
46. Sjøgren, B., and Svedberg, T.: *J. Am. Chem. Soc.*, **52**, 3650 (1930).
47. Hitchcock, D. I.: *J. Gen. Physiol.*, **6**, 457 (1924).
48. Gavrilov, N. J., and Simskaija, A.: *Biochem. Z.*, **238**, 44 (1931).
49. Morse, W.: *Collagen, Applied Biochemistry* (1927).
50. Marriott, R. H.: *J. Soc. Leather Trades Chem.*, **10**, 132 (1926).
51. Kuentzel, A., und Dietsch, O.: *Biochem. Z.*, **231**, 435 (1931).
52. Sadikof, W. S.: *Z. physiol. Chem.*, **39**, 396 (1903).
53. Abderhalden, E., und Schmitz, A.: *Z. physiol. Chem.*, **190**, 101 (1930).
54. Knaggs, J., and Schryver, S. B.: *Biochem. J.*, **18**, 1095 (1924).
55. Morse, W.: *Ind. Eng. Chem.*, **23**, 1471 (1931).
56. Gerngross, G. et al.: *Collegium*, Nr. **724**, 338 (1931).
57. Theis, et al.: *J. Am. Leather Chem. Assoc.*, **27**, 12 (1932).
58. Smith, C. R.: *J. Am. Chem. Soc.*, **41**, 135 (1919).
59. Shuravliev, D., and Arbusov, D.: *Organ of the All-Russia Leather Syndicate*, No. **12**, 41 (1926).
60. Bechold, H., und Neumann, S.: *Z. angew. Chem.*, **37**, 534 (1924).
61. Stohmann, F., und Langbein, H.: *J. f. prak. Chem.*, (2) **44**, 336 (1891).
62. Brown, H.: *J. Biol. Chem.*, **68**, 729 (1926).
63. Hasselbalch, K. A.: *Skand. Arch. f. Physiol.*, **25**, 55 (1911).
64. Lucas, N. S.: *Biochem. J.*, **25**, 57 (1931).
65. Dejust, L. H.: *Bull. soc. chim. biol.*, **9**, 1165 (1927).
66. Loewe, A., und Cronheim, G.: *Biochem. Z.*, **256**, 411 (1932).
67. Wahlgren, V.: *Arch. f. exp. Path. u. Pharm.*, **61**, 97 (1909).
68. McLaughlin, G. D., and Theis, E. R.: *J. Am. Leather Chem. Assoc.*, **19**, 428 (1924).
69. Nadel, A.: *Biochem. Z.*, **249**, 83 (1932).
70. Kooyman, D. J.: *Proc. Soc. Exptl. Biol. Med.*, **29**, 485 (1932).
71. Klose, E.: *J. f. Kinderheilkunde*, **91**, 157 (1920).

72. Unna, P. G., und Golodetz, L.: *Biochem. Z.*, **20**, 469 (1909).
73. Eckstein, H. C., und Wile, U. J.: *J. Biol. Chem.*, **69**, 181 (1926).
74. Hentschel, H., und Schindel, L.: *Klin. Wochschr.*, **9**, 262 (1930).
75. Urbach, E., und Rejtő: *Klin. Arch. f. Derm. u. Syph.*, **166**, 478 (1932).
76. Folin, O., Trimble, H. C., and Neuman, L. H.: *J. Biol. Chem.*, **75**, 263 (1927).
77. Ellinger, P.: *Münch. med. Wochschr.*, **61**, 2336 (1914).
78. Milbradt, W.: *Biochem. Z.*, **223**, 278 (1931).
79. Noback, C. V., and Modell, W.: *Zoologica* (New York Zoological Society), **11**, 19 (1930).
80. Turley, H. G., and Morse, W.: *J. Am. Leather Chem. Assoc.*, **27**, 282 (1932).
81. Martin, S. H.: *China Med. J.*, **45**, 244 (1931).
82. Schultz, W.: *Arch. f. Derm. u. Syph.*, **165**, 405 (1932).
83. Beadles, J. R., Braman, W. W., and Mitchell, H. H.: *J. Biol. Chem.*, **88**, 623 (1930).
84. Seymour, R. J.: *Am. J. Physiol.*, **78**, 281 (1926).
85. Wilson, J. A.: *The Chemistry of Leather Manufacture* (1929).
86. Marchionini, S.: *Klin. Wochschr.*, **8**, 924 (1929).
87. Fishberg, E., and Bierman, W.: *J. Biol. Chem.*, **97**, 433 (1932).
88. Thenard, L. J.: *Chimie* (1833).
89. Gamble, J. L., Ross, G. S., and Tisdall, F. F.: *J. Biol. Chem.*, **57**, 633 (1923).
90. Saiki, A. K., Olmanson, G., and Talbert, G. A.: *Am. J. Physiol.*, **100**, 328 (1932).
91. Marshall, E. K.: *J. Biol. Chem.*, **15**, 493 (1913).
92. Talbert, G. A., Finkle, J. R., and Kalsuki, S. S.: *Am. J. Physiol.*, **82**, 153 (1927).
93. Talbert, G. A., and Haugen, C. O.: *Am. J. Physiol.*, **81**, 74 (1927).
94. Talbert, G. A.: *Am. J. Physiol.*, **63**, 350 (1922-23).
95. Haugen, C. O., and Talbert, G. A.: *Am. J. Physiol.*, **85**, 224 (1928).
96. Silvers, S., Forster, W., and Talbert, G. A.: *Am. J. Physiol.*, **84**, 577 (1928).
97. Talbott, J. H., Foelling, A., Henderson, L. J., Dill, D. B., Edwards, H. T., and Bergren, R. E. L.: *J. Biol. Chem.*, **78**, 445 (1928).
98. Darmstaedter, A., und Liftschuetz, S.: *Ber.*, **28**, 2133; **29**, 618, 1474; **31**, 97 (1895-1898).
99. Lamois, A., und Martz, S.: *Zentralb. f. med. Wiss.*, Jh. 1898, p. 1.
100. Oppenheimer, C.: *Handb. d. Biochemie*, **5**, 416 (1925).
101. Lehmann, C. G.: *Ber. d. säch. Akad. Wiss.*, **2**, 1 (1869).

CHAPTER XXVIII

URINE

IN animals like the earthworm, where the body consists of a series of segments, a pair of tubules known as *nephridia* serve as kidneys in that they drain the body fluid from the interior of the segment to the outside. In man, two things have modified this simple arrangement; first, the restriction of the body cavity proper to relatively small sacs in different portions of the body; and, secondly, the agglomeration of the tubules, or their respective ducts, into what we call the kidney. The adult kidney results from a series of developments and suppressions of the segmentally arranged tubules. Thus, the earliest portion of the kidney to form in man is the pronephros, or anterior part of the tubule system. Thus far there is no connection with the blood system, but in the growth of the tubules farther towards the rear of the embryo (mesonephros), a tuft of arterioles comes into intimate contact with the mouths of the tubules in the so-called "malpighian capsule." The ducts are coiled. The pronephros already has developed a longitudinal duct for bearing the secretion of the several tubules, rather than pouring the urine to the outside through each individual tubule, and the mesonephros appropriates this duct for its own use. The pronephros then atrophies.

The third and last portion of the tubule system forms from the mesonephros. It is simply the portion lying more posterior than the pronephros. The tubules develop as outpushings of the mesonephric duct (the old pronephric duct). A portion of this duct, due to the fusing of terminal branches, forms the pelvis of the kidney and its extension posteriorly is the ureter. In the metanephros, all connections with the body cavity are obliterated and the adult kidney, then, becomes wholly an organ for excreting the constituents of the blood that are passed into the urine. The problem of the chemist, then, is to determine what substances are thus excreted and what their relations are with respect to the precursors in the blood and in metabolism.

Renal Dynamics.—What factors permit the urinary constituents to pass into the renal system from the blood? Obviously, two explanations are possible; first, that the living membrane may exert some specific selective permeability for constituents of the blood ("secretion theory"); or, secondly, the process may be purely mechanical, substances being excreted from the blood into the tubules, some being resorbed into the circulation in the glandular portion of the tubule, in order that the concentration of the urine in any constit-

uent be maintained within narrow limits (filtration theory). The signal work of Richards^{1, 2, 3, 4, 5} following the development of a method for collecting fluid from the glomeruli⁶ has gone far to prove that the latter mechanism is adequate. The evidence is based on the proof that in the serum of the blood and the urine of the glomerulus the urea concentration is the same, the electrical conductivity is identical and when dyes are added, and permitted to pass into the glomerulus, after a time the concentration is the same. Others have shown that the chloride content of serum and urine is the same.⁷ There are discrepancies, however, such as Wearn and Richards' estimate of chloride concentration;⁶ and yet reexamination of some points, such as those of molecular concentration by White,^{3, 8} have removed difficulties. Taken as a whole, the burden of proof lies upon the advocates of the secretion theory. We may conclude, then, that modern investigations approve the older hypothesis of Carl Ludwig⁹ for urine separation from blood serum; namely, by the principles of hydrostatics, which depend upon the factors affecting permeability, membrane equilibria and other physical and chemical factors.

Chemical Composition of Urine.—All inorganic constituents of blood plasma are found in the urine. Food inorganic substances are absorbed and an optimum is retained in the blood and organs, the excess being eliminated through the kidneys. The amount excreted, therefore, is related to the need for inorganic substances in the blood and tissues; and this again depends upon the uses to which these substances are put. We may summarize the needs for inorganic salts and other substances in the body: (a) As solvent; (b) as a factor in maintaining the proper pH; (c) as detoxicant; (d) as a factor in oxidation-reduction phenomena; (e) as a factor in maintaining such functions as rhythmic movements of heart, cilia, etc.; (f) to serve in respiratory functions as transfer agents; (g) as a constituent of architectonic structures such as bones; (h) as a catalyst for reactions that otherwise would proceed too slowly; (i) in maintaining turgescence of cells and tissues; (j) perhaps as harmonic factors, either as hormones or accessory to such substances.

These substances act in equilibria, sometimes quite complex, involving not only other salts, but organic compounds as well.

In lower organisms, like sea worms, bathed continually in sea water, the nephridia play a far less important rôle than in the higher forms with air as the external medium, where the nephridia are immediately associated with the blood system. However, not even in man is the kidney unique as an excretory organ. The skin passes sodium chloride, urea and other substances, and lime is a characteristic of feces. Moreover, the effluvia from the skin (dried cells, hair, nails) afford a means of excreting certain substances. With regard to nitrogen, however, the kidneys are overwhelmingly important, indeed so much so that in metabolic work the nitrogen excreted through any other channel is negligible.

Chlorine.—This halogen occurs in the urine solely as chloride,²¹ of which sodium chloride occurs in the greatest amount (about 15 Gm. per twenty-four hours). Of the different chlorides, that of sodium is excreted promptly.²³ Thus when 0.1 Gm. sodium chloride per kilo of body weight is injected intravenously, all is passed within a half hour, as contrasted with CaCl_2 and MgCl_2 , which require four hours. Again, there seems to be a selective permeability for sodium chloride and sodium bromide, for when these salts are injected the chloride ion is excreted promptly whereas the bromide ion is retained.^{16, 25} The chlorides are directly related to water content of tissues and urine. Deprivation of salt causes decrease in urine volume even to total anuria.¹⁷ Water is not retained by tissues unless they are supplied with chloride. In normal subjects, but not in those with renal involvement, the expression for the relation between chloride excretion and blood (serum) chloride concentration of Ambard¹⁸ holds within statistical limits. However, it is the amount of chloride held by the tissues rather than that held by the blood that determines the rate and amount of chloride excretion. Flushing the body with water exerts a "wash-out" action, although the tissues retain chlorides tenaciously. A limit, however, in this action is reached when the urine volume exceeds 300 cc. per hour; then the chlorine falls. Hubbard¹⁴ found no correlation between urine chloride concentration and the period of greatest loss of chloride during the postprandial period known as the "alkaline tide," when chloride as hydrogen chloride (HCl) is being secreted by the gastric mucosa.

The urine is much richer in chloride than the perspiration.¹⁵ No direct correlation exists between urine and sweat chloride.²⁴

In total solids, the urine concentration is five times that of the perspiration. When there is excessive loss of chloride in the perspiration, there is a corresponding lowering of chloride excretion in the urine; in other words, there is no compensatory action among the various organs of excretion.

However, there is a highly delicate compensatory reaction in the blood, tissues and kidneys for regulating the osmotic pressure and acid-base balance, which may be represented by the normal minimal chloride concentration in the blood (plasma) of about 100 millimoles. Below that figure, chloride excretion in the urine is suppressed. The urine, of course, is but one of two means of escape of plasma chloride, the other being into the tissue spaces; but apparently, the same regulatory mechanism holds for the tissues in general as for the kidney, at least in principle. This is seen in an interesting way in pathologic cases such as, for example, in nephritis, which involves edema. While edema is pathologic, nevertheless, there seems to be a gradual transition between the normal tissue's capacity for regulating saline and water content and the abnormal ability of the nephritic, for when put to the pace by administering massive doses of salt to a normal person (40 Gm.), edema developed due to the storage of both salt

and water.²⁶ The amount of salt excreted bears a direct relationship to the degree of saturation of the tissues for salt, for in a subject who has been deprived of salt, the amount necessary to be completely excreted in twenty-four hours when given by mouth or by injection, is much greater than in the case of a man who has had his complement of salt. This is an inhibiting factor in de Wesselow's test.²⁷

The diuretic action of sodium chloride is less than that of some other salts, such as $MgCl_2$, when given by mouth. When Epsom salt is administered by mouth, chloride is displaced from the serum and migrates into the tissues, while the sulfate ion is excreted into the urine. The loss of chloride, then, after $MgSO_4$ catharsis, is purely mechanical, the "metabolic" chloride being retained in the tissues.

Chloride is important in regulating the water economy of the body and in relation to acid-base balance.

Potassium.—Potassium is characteristic of cells and tissues, as contrasted with sodium, which is typical of body fluids. This distinction appears in fasting states, or in others in which there is a considerable loss of tissue; here there is an increase in the ratio K/Na due to the withdrawal of potassium from the wasting tissues.²⁸ As an example of the different distribution of K and Na , we cite the concentration of potassium in blood serum as 19 mg. per 100 cc. of serum contrasted with about 400 mg. per 100 cc. of centrifuged cellular elements (erythrocytes; leukocytes).²⁹ Potassium chloride is excreted more quickly than sodium chloride.³⁰ For this reason, potassium salts are not the cause of much edema, which depends upon retained salts.³¹ Potassium chloride is a marked diuretic and when administered by mouth may cause retention of sodium in man,³² while in rats there may be an increase in sodium excretion.³³ Such diuresis may cause the loss of endogenous potassium.³⁴ The loss of sodium in rats is attributed to the greater diuretic effect of potassium over sodium. If there is a deficit of sodium, and potassium chloride be given by mouth, there is little or no loss of sodium, and *vice versa* for potassium.³² Water is retained in these cases in the tissues.³² In describing chlorides in the body we referred to perspiration, and we may say here that about half of the chloride in perspiration (41 per cent) is in the form of potassium chloride.⁵

Calcium.—Calcium has a dual means of leaving the body: (a) By way of the feces (65–75 per cent), and (b) *via* the urine (25–35 per cent). In infants even less is excreted in the urine under normal circumstances (5 per cent).³⁷ The reaction of the intestine is an important factor in determining which avenue is to be the more important. An acid reaction in the intestine, or a true acidosis in the organism leads to augmented absorption of calcium from the intestine and hence elimination of the substance *via* kidney rather than by bowel.³⁸ Zucker³⁹ found that when 300 cc. of decinormal HCl was added to the experimental animal, 32 per cent of the calcium was eliminated through the urine; $NaHCO_3$ (15 Gm.) caused 22 per cent of

the calcium to be eliminated in the feces. Such observations favor the use of CaCl_2 over other forms of calcium (such as the lactate and gluconate), for calcium is absorbed more readily from the intestine in the acid reaction set up by the chloride ion. Too much acid may lead to depletion of base and reduction of amount of lime through excessive elimination of calcium in the urine.

Magnesium.—From 20 to 50 per cent of the magnesium salts ingested is excreted in the urine in infants and adults. As in the case of calcium the major portion is excreted *via* feces. Calcium and magnesium then share the property of being eliminated in both urine and feces, while sodium and potassium pass off chiefly in the urine. Excess administration of Mg may lead to disturbance in the solubilities of magnesium in the urinary tract and calculi may develop.⁴⁰ Magnesium seems to be correlated with phosphorus metabolism. Thus Euler⁴¹ suggests that it is required in phosphorulization. Hence, it may not be a relation to calcium that is frequently suggested, but one to phosphorus. Low magnesium content of food (1.8 parts per million) produces what McCollum and colleagues call "magnesium tetany," in which no magnesium is excreted save in the feces.⁴²

Phosphorus.—Contrasted with calcium and magnesium on the one hand and sodium and potassium on the other, phosphorus is excreted largely in the urine, but to a considerable extent (30 per cent) in the feces also. There seems to be a fairly close quantitative relationship between intestinal and urinary excretion of phosphorus, for conditions that favor excretion by one avenue contribute to lessen the excretion by the other.⁴³ As in the case of calcium, acid tends to favor urinary excretion of phosphorus, because it aids absorption from the alimentary tract. High fat diets cause withdrawal of ingested phosphorus from absorption and hence a lowering of urinary excretion of phosphorus.⁴⁴ On the other hand, fasting, inducing tissue destruction, favors increased urine excretion of phosphorus.

As phosphoric acid, phosphorus is excreted in amounts of about 1 to 5 Gm. in twenty-four hours. The precursor of phosphate in the urine is not known, but urinary phosphorus exists almost entirely as inorganic phosphate.⁴⁶ The great reservoir of phosphorus is the tertiary salt of phosphoric acid, $\text{Ca}_3(\text{PO}_4)_2$ of bone; the soluble phosphates of the blood are mixtures of primary ($-\text{H}_2\text{PO}_4$) and secondary ($-\text{HPO}_4$) phosphates, the proportion of which is regulated largely by the hydrogen ion concentration of the blood (serum).⁴⁷ Organic phosphate exists in the form of lipoid phosphate (as in lecithin), phosphoric acid of nucleotides, hexosephosphate of muscle, phosphocreatine of muscle, or phospho-arginine. Another form of phosphorus is pyrophosphate.⁴⁸ A parallelism exists between plasma phosphate and urinary phosphate.⁴⁹ While the locus of such transformations is outside the urinary tract, the interchange of acidic and basic phosphate is a function of the kidney. This function has to do with rendering of the urine acid by virtue of the sparing of basic

phosphate for the alkali reserve of the blood system, for the acidity of the urine is determined largely by the content of $\text{—H}_2\text{PO}_4$.⁴⁷

Phosphaturia is a much misused term. Properly it refers to an acceleration of excretion of phosphate, such as in experimental feeding of ammonium chloride, which reduces the pH of the serum and favors the urinary excretion of phosphate, without changing the blood level of phosphate concentration.^{50, 51} In general, true acidosis is conducive to phosphaturia.^{47, 50} On a high vegetable diet and low nitrogen, an alkaline urine is produced which is favorable to the precipitation of phosphates as an evident cloud; frequently such a condition is referred to as "phosphaturia." In our own experience, urates make up the large part of such a precipitate. The interreactions of acids in the body and the reflection of their metabolism in the acid content of the urine is shown when sulfuric acid is administered by mouth.⁵² Sixty-one per cent of the 300 cc. of equivalent normal H_2SO_4 fed to pigs resulted in being neutralized by ammonia; 5 per cent was accountable as excreted phosphoric acid in the urine.

The pituitary gland, aside from its relations to water balance, seems to exert some control over phosphate excretion, as Robison and colleagues⁵³ assert, for damage to the pituitary inhibits phosphate excretion *via* kidney. Injection of glycerophosphate at this point causes phosphate excretion, which points to the presence of a phosphatase of nature similar to the well-known Robison enzyme of cartilage, but located in the kidney.^{54, 58} It appears, then, that the urine phosphate is derived rather from organic than inorganic phosphate, which, injected in place of the organic phosphate, leads to much less phosphate excretion.⁵⁷ In fairness to Kay, we do not have his sanction to this interpretation, for he believes the phosphate excreted after glycerophosphate injection is of an organic nature.⁵⁵

The kind of diuretic used seems to play a decided rôle in modifying phosphate excretion. Thus, caffeine and other methylated purines diminish urinary phosphate excretion,⁵⁹ while saline diuretics and urea induce increased phosphate excretion.⁶⁰ There seems to be no "wash-out" phenomenon whereby simple administration of water produces a modification of phosphate excretion.

Sulfur.—Unoxidized sulfur or neutral sulfur has in some way escaped oxidation. Such sulfur represents biliary constituents (bile salts, taurine) and in part cystine.⁶¹ When cystine and cysteine are administered to a dog, 70 per cent is excreted as oxidized sulfur (sulfate) and 4 per cent as neutral or unoxidized sulfur. Cystine in combined form, such as glutathione, affords slightly more unoxidized sulfur (10 per cent). Brand⁶² has suggested that cystine is metabolized not as such, but in some complex, and gives evidence that in the disease cystinuria, Delépine's older view⁶³ of a cystine complex is probably correct.

Some of the unoxidized sulfur comes from the urinary pigment, urochrome, constantly excreted into the urine.⁶⁴ Cystine itself seems

to be correlated with the standard metabolism, for Looney and colleagues found it to be present in human urine of normal adults at about 30 mg. per twenty-four hours.⁶⁵ The taurine of urine doubtless is of exogenous origin, being taken up from the intestine and excreted into the urine.⁶⁶ A small amount of thiocyanate occurs in the urine, probably having an origin similar to that of oral thiocyanate.⁶⁷

Oxidized Sulfur, Sulfate Sulfur.—These are simple inorganic sulfates, chiefly those absorbed as such from the intestine, but also compounds that are converted in the body (chiefly in the liver) into sulfates (like sulfhydryls, H_2S , mercaptans, etc., derived from intestinal putrefaction products).

The **etheral sulfates**, representing another form of oxidized sulfur, are compounds of sulfuric acid or its equivalent, in the form of esters. A familiar example is indoxyl, a phenol, which is conjugated with sulfate (potassium salt), the phenol arising among the putrefactive products of protein containing the amino acid, tryptophan, an indole substance. The synthesis of etheral sulfates is due, in part at least, to sulfates of metabolic origin in the body proper and not to those included with food or arising from oxidation in the intestine.⁶⁹ The locus of etheral sulfate synthesis seems to be in the liver.⁷¹ Miss Denis found that not all urinary sulfur comes from metabolic sources; when sulfur was fed in amounts of 0.5 Gm. per kilogram of body weight, per twenty-four hours, 10 per cent appeared in the urine, this being due to oxidized hydrogen sulfide arising in putrefaction in the intestine;⁷² and Hele⁷³ recently has given evidence that exogenous sulfate is used to a considerable extent in the synthesis of etheral sulfates; for orally administered, Na_2SO_4 is utilized along with phenol and indole in the formation of such sulfates.

The *sulfur-nitrogen ratio* is important in any metabolic studies, for it affords a means of evaluating observations on the manner in which protein is metabolized in intermediate processes. Folin⁷⁵ early distinguished the invariable, endogenous metabolism from the variable, exogenous form. The former was characterized by excretion of creatinine, neutral sulfur, uric acid and etheral sulfates in fairly constant amounts for the given individual and for physiologic states of reasonable normality; while correspondingly, urea and inorganic sulfates belong with the variable form of metabolism (exogenous). Sulfur in the form of neutral (unoxidized) sulfur and also etheral sulfates seems to remain quite constant under conditions of profound changes in metabolism, such as rest and work.⁷⁶ Benedict noted a decrease in unoxidized sulfur during fasting;⁷⁷ other workers a rise in sulfur during work and in rest, especially when compared with the excretion of nitrogen.^{78, 79} The increase in sulfur was far the greatest in inorganic sulfur. Etheral sulfates decreased, unoxidized sulfur remained constant.

The sulfur-nitrogen ratio (S/N) is of value to the student of metabolism in determining the character of the protein that is undergoing

metabolism. In 1881, in Hermann's *Handbuch der Physiologie*, Carl Voit⁸⁰ proposed a nomenclature for protein as follows: Circulating protein, labile and poor in sulfur; retained, stable protein, rich in sulfur. More recently Wilson⁸¹ modified this scheme as follows: Circulating protein in Voit's sense; transitional protein, intermediate in sulfur content between the labile form just mentioned and the stable protein. The sulfur content is level with that in the tissues, especially in muscle. The higher the sulfur content the more stable the protein.⁸² It is the sulfur of the labile protein that first appears in the urine after injury or fasting.⁷⁶ The "basal" sulfur-nitrogen ratio approximates 1:18.5. Feeding meat following four days on a basal diet consisting of bread, butter and fruit, the S/N ratio was 1:15. Doubling the quantity of meat gave relatively greater sulfur (1:14.2) and again, reducing the amount of meat to one half, gave less sulfur (1:16.3). In the pathology of renal disease, such as nephritis, these studies are of marked importance. They also permit the estimation of how much sulfur is removed from proteins. Thus it has been shown that about 90 per cent of the sulfur in the ratio 1:14.5 is of inorganic nature (sulfate). The average nitrogen excretion is about 14 Gm. per twenty-four hours, corresponding to 88 Gm. of protein. This involves 6 Gm. of sulfur, or about 500 cc. of decinormal H_2SO_4 excreted in a day.

Other Mineral Constituents of the Urine.—The following inorganic substances have been detected in human or animal urine: Aluminum, arsenic, boron, chromium, copper, fluorine, iron, iodine, cobalt, lithium, manganese, nickel, rubidium, silver, strontium, titanium, vanadium, zinc, tin, in addition to those mentioned before.⁸⁵ Zinc has been identified by Fairhall.⁸⁸ Copper is discussed by Fox and Ramage.⁸⁹ Manganese is stored in the kidney.⁹⁰ Boron, zinc and manganese are derived largely from dicotyledonous plants. Zinc occurs in wheat and barley.⁹¹ About 50 mg. of iodine occurs in the human body.⁹² The daily loss *via* the kidney is extremely small, the blood iodine being from $11-16 \times 10^{-6}$ Gm. per 100 cc. of blood.

Urea.—Of the organic constituents, urea is by far the most important whether from the standpoint of quantity or of function; for nothing reflects the state of metabolism more certainly than this substance.

The average dry weight of urea excreted by the adult human subject in twenty-four hours is 30 Gm. This varies with physiologic conditions. Van Slyke has shown the close relation between blood urea and that excreted through the kidneys.⁴³ The marked diuretic property of urea is a factor in urea excretion, which becomes less effective as the amount passing through the kidney into the urine becomes restricted. Austin, Stillman and Van Slyke⁹¹ call this limit the "augmentation limit," and quantitatively it is about 2 cc. of urine per minute. Below this limit, the excretion of urea ceases to be a linear function with respect to blood urea and approaches the square root of the volume of urine passing through the kidney. Above the

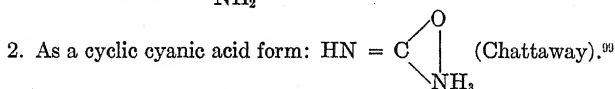
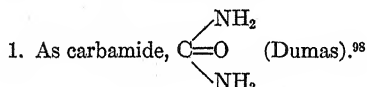
"augmentation limit," urea is excreted directly in proportion to the urea in a given volume of blood which passed through the kidney from the renal artery. This volume is approximately 75 cc. per minute, as a maximum, or, as the investigators themselves speak of it, the "maximum clearance" of urea from the blood.

Urea crystallizes as snowy masses of tetragonal crystals, very soluble in water. One Gm. of water at 5° C. dissolves 0.77 Gm.; 1000 Gm. at 17° C. and 1094 at 20° C. It is soluble to the extent of 20 Gm. in 100 cc. of ethyl alcohol. It is slightly soluble in ethyl ether and is capable of being sublimed *in vacuo*. The crystals melt at 132.7° C. The taste of pure urea varies with the individual, some finding it bitter, others tasteless or salty.

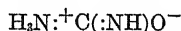
Urea was discovered by Rouelle⁹³ in urine, but Prout⁹⁴ first analyzed it. Writing to Berzelius in 1825, F. Woehler said: "I can make urea without calling on my kidneys";⁹⁵ and two years later⁹⁶ he announced "my research gave the unexpected result that by the combination of cyanic acid with ammonia, urea is formed." This is known as "Woehler's synthesis" and is referred to as the first synthesis of an organic compound—which is not true.⁹⁷

The present state of our knowledge of the chemical structure of urea is given by Sir F. G. Hopkins: "It is certainly instructive to realize that the molecular structure of a substance already synthesized one hundred years ago, a substance with a molecular weight of not more than 60, is yet a matter of debate."⁹⁸

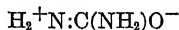
Two chief concepts concerning the space structure of urea exist:



The electronic properties of urea have been assigned as follows:¹⁰⁰ As "zwitterion," the polar formula in water is:



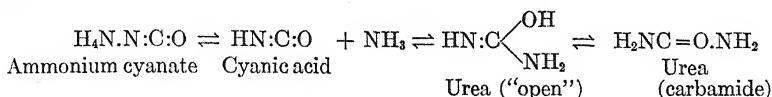
or, by a somewhat different arrangement:¹⁰¹



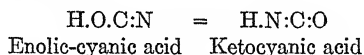
but an objection has been made¹⁰² to a polar formula for urea because analysis reveals no such form.¹⁰² A "zwitterion" requires the product of both dissociation constants involved to be greater than 10^{-16} , which is not realized in polar formulas for urea; and lastly, the crystal molecule is symmetrical and does not show structural change in an aqueous medium.

As to the merits of the carbamide formula versus the cyanic acid form, Werner⁹⁷ being the chief advocate, we may again quote Sir F.

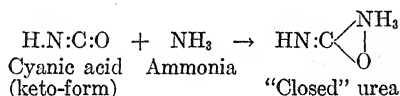
G. Hopkins: "I think we should be grateful to Professor Werner for the 'ring' and 'open' structural formulas of urea, even if their reality is not yet proven. I think that carbamide may not be absent from the laboratory of living tissues. After all, ammonia *does* meet with CO_2 there and the liver *can* dehydrolyze ammonia carbonate."⁹⁵ Werner⁹⁷ following Chattaway's⁹⁹ explanation of Wöhler's synthesis, represents the reactions thus:



in which cyanic acid is in equilibrium with ammonia and these in turn with urea. The synthesis of urea might occur in the tissues in this manner; for if ammonia be removed from the cyanate, an enol form of cyanic acid results:

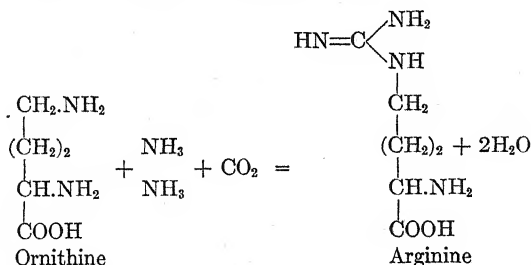


When ammonia is added we have urea, but not in the carbamide form:

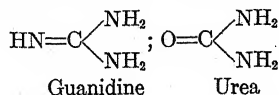


Returning, now, to the carbamide formula, we should expect urea to show amino reactions if its formula is that of a diamino compound, $\text{H}_2\text{N}-\text{CO}-\text{NH}_2$; yet pure urea and pure nitrous acid do not react¹⁰³ as amino compounds and this casts serious doubt upon the validity of the formula. If a small amount of mineral acid be admitted into the reaction, then cyanic acid can be identified by its silver salt. (See the monograph by Werner.⁹⁷)

Krebs has obtained evidence for the origin of urea through the amino acid ornithine acting with NH_3 and CO_2 as follows:¹⁰⁴



Arginine, then, bearing a guanidine group analogous to urea,



is hydrolyzed to ornithine and urea (reversal of the above equations).

This is accomplished by an enzyme in the liver, arginase. Moreover, an amino acid standing between urea and guanidine, citrullin,¹⁰⁵ behaves as an intermediate in the reaction, being synthesized from ornithine, ammonia and CO_2 when introduced into a liver hash from which ammonia has previously been excluded and admitted only slowly. Werner's statement⁹⁷ (page 75) that "the production of urea from guanidine and arginine has not yet been properly investigated" is no longer true. While urea formation seems to be relegated to the liver,¹⁰⁶ the embryonic kidney¹⁰⁷ (but not the adult kidney¹¹⁵) may produce urea. If Kreh's view is correct, we understand why the liver is alone in synthesizing urea, for arginase occurs in copious amounts in the kidney,¹⁰⁸ but it is improbable that extra hepatic urea synthesis is of metabolic importance.¹³⁶

Folin, in a classical investigation,¹⁰⁹ showed that urea was excreted proportionately to other nitrogenous compounds much more liberally on a high protein diet (total nitrogenous excretion 16.8 Gm. per twenty-four hours) than on a low protein diet (3.6 Gm. per twenty-four hours); the urea, estimated as urea nitrogen being 14.7 Gm. (87.5 per cent) on high protein intakes as against 2.2 Gm. (61.7 per cent) on the low nitrogen diet (composed chiefly of sugars, starches and fat). The source of urea may be metabolized body protein, as in fasting; but the excretion of urea in starvation varies with another factor—acidosis; and this in turn causes the formation of ammonia which affects the amount of urea excreted. But prior to acidosis there is a rise in blood urea¹¹⁰ and immediately before death, another rise in urea occurs, presumably because of renal failure; here urea is not excreted. Urea, in normal subjects, is not promptly excreted after administration;^{111, 112} but apparently the urea is not retained as such, but in another form. When amino acids are administered urea is not promptly excreted.¹¹³

In the resolution of exudates and other instances of autolysis, urea takes its origin from arginine.¹¹⁴ The great rise in accumulation in nitrogen during intestinal obstruction does not involve urea.¹¹⁶

Urea administered by mouth (15 Gm. in 100 cc. H_2O) and correlated with urea excretion during the following period of two hours, has served as a rapid means of determining the function of the kidney.^{17, 117} A normal kidney concentrates urea over 2 per cent above the original level in such cases. Such a procedure is much simpler than the method of Ambard,^{18, 118} although there is a correlation made in Ambard's coefficient with blood urea,

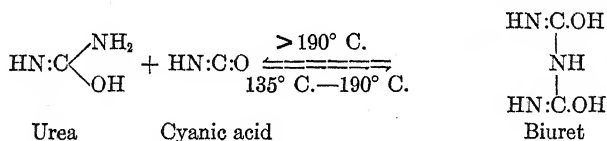
$$\text{Urea coefficient} = \frac{U^{0.75}}{B} \sqrt{V}$$

where U is the concentration of urea in the urine excreted, B the corresponding concentration in the blood and V the volume of urine. This volume relation, however, holds for excretion of urine below 2 cc.

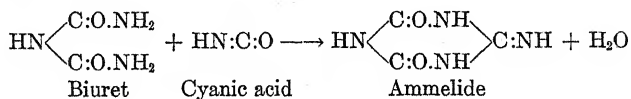
per minute; above that "augmentation limit," the volume, not the root, is employed directly. Moreover, as has been stated, it is not necessary to use the power of urine-urea-concentration, but simply the quantity *U* itself. Finally, the Maclean index⁷ is derived from Am-bard's formula by removing the radical, inverting the fraction and, by employing a numerical constant, bringing the urea secretory index to 100 for normal subjects.

In bacteria a urea-splitting enzyme was early discovered.¹¹⁹ Since then it has been identified in leguminous seeds, especially of the soy bean,¹²⁰ from which it is easily extracted by water. Urease affects urea only in a free condition,¹²¹ and from the extensive study of Fearson,¹²² the action of urease on urea is as follows: (a) Condensation of urea on the urease; (b) formation of NH_3 and cyanic acid by dissociation, the ammonia combining chemically with the urease, the cyanic acid being hydrolyzed by water present in the reaction. Thus, contrary to many statements,¹²³ there is no "hydrolysis" of urea by urease. The essential process is a dissociation of urea into ammonia and cyanic acid, the "hydrolysis" involving a secondary change of the cyanic acid.

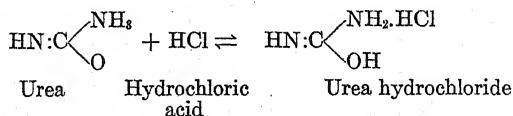
When urea is heated in the absence of water to about 150°C . (but to not more than 190°C .), the following reaction occurs:



In the *biuret test* for protein, urea plays no part. Cyanic acid arises from amino acids and a violet color is obtained in the presence of copper hydroxide. Biuret decomposes into ammelide in the presence of cyanuric acid:

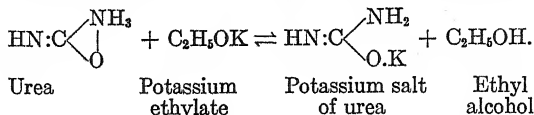


Salts with definite crystal forms are produced when urea is treated with mineral acids. Thus, HCl and urea react to produce urea hydrochloride:

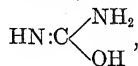


Urea nitrate crystals have long been used to identify urea. In acid solution, urea is very stable. Werner⁹⁷ kept a 0.2N urea solution in normal HCl at 20°C . for six weeks without change in the titer of the solution. Oxalic acid forms urea oxalate in the presence of urea.

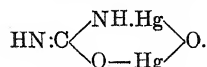
Heated in a sealed tube with alcoholic KOH, urea is broken down to KCNO, NH_3 and H_2O ; but at 230°C . the reaction is as follows:



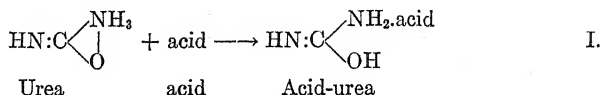
Silver forms a decomposition product of urea in the presence of caustic alkali, which is necessary to decompose the urea. Thus, ammoniacal silver nitrate, acting with KOH, forms first,



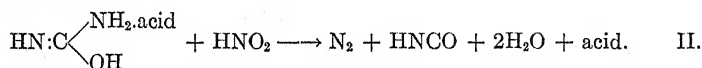
the open form of urea; then $\text{HN:C} \begin{array}{c} \text{NH.Ag} \\ \diagup \quad \diagdown \\ \text{O.Ag} \end{array}$. Similarly, mercury forms



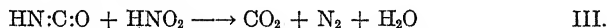
With nitrous acid a salt is first formed, since pure HNO_2 does not react with urea:



Such an acid is the acetic acid used in the gasometric method of Van Slyke, which employs the organic acid to react with NaNO_2 and produces nitrous acid involved in the gasometric reaction. Next, the nitrous acid in the deamination of urea reacts with the compound in (I) as follows:

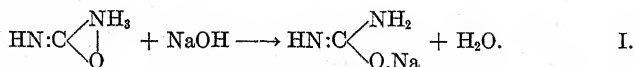


The cyanic acid also reacts:

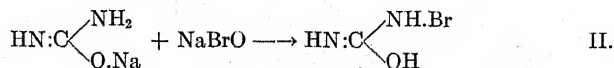


In Van Slyke's procedure the CO_2 is removed by alkali in the Hempel pipet and the N_2 is read over water in the buret. One nitrogen is furnished by urea and one by the nitrous acid, and thus the volume of nitrogen in the nitrometer must be halved in order to give the quantitative value desired.

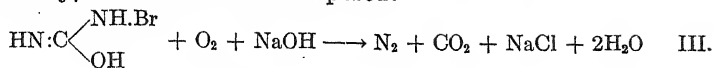
As in the case of nitrous acid, urea is not affected by pure hypobromite (or any hypohalogenite), and hence it must first have its cyclic portion opened. This is accomplished by the strong caustic usually employed:



Next, bromo-urea is formed:



Finally, bromo-urea is decomposed:



Xanthidrol, $\text{HCOH} \begin{array}{c} \text{C}_6\text{H}_4 \\ \diagup \quad \diagdown \\ \text{O} \\ \diagdown \quad \diagup \\ \text{C}_6\text{H}_4 \end{array}$ is widely used in precipitating urea from

solutions, owing to its high insolubility.¹²⁴

In birds, uric acid, rather than urea is the chief nitrogenous excretion product. It has been suggested that the high solubility of urea is undesirable in birds in flight and that uric acid is better adapted to the stability desired; but the whole group *Sauropsida*, to which both reptiles and birds belong, has uric acid as the characteristic excretory product of the kidneys.

Ammonia.—Ammonia is essentially an excretory product, occurring, as nitrogen, on the average of 0.75 Gm. per twenty-four hours (2.5 per cent of the total urinary nitrogen excretion). Very little ammonia is found in the blood owing to the rapid withdrawal by the liver. It occurs chiefly in the portal and peripheral circulation. The portal circulation receives its ammonia from the intestine, where it arises from bacterial deamination of food materials; and from the blood itself, being excreted into the alimentary tract; and from gastric secretions, under the action of a gastric urease found in the mucosa.^{125, 129} Urinary ammonia exists largely as ammonium hydroxide or carbonate and may readily be aspirated from the urine by a strong air blast after previous alkalization of the urine. It is formed in the kidney^{126, 128} from urea,¹²⁷ amino acids^{134, 135, 136, 137, 138} and perhaps from other urinary nitrogenous substances like nucleotides.¹²⁹ It acts like a base, replacing other bases of value in the alkali reserve, such as K, Na, Mg, Ca. It is doubtful that a quantitative relation between urine reaction and ammonia content of urine can be demonstrated.^{130, 131, 132, 133} The total, or titratable acidity of the urine bears perhaps a closer relation;¹³¹ and Van Slyke¹⁴⁰ finds the normal ratios, $\frac{\text{decinormal ammonia}}{\text{decinormal total acid}} = > 1$, usually 0.7 to 2.8.

Amino Acids.—These compounds occur to a slight extent in normal urine (about 1 per cent total urinary nitrogen). The fact that they are increased in excretion when more water is flushed through the kidney seems to indicate that the small amount of amino acids comes from the blood, where they circulate to an extent of about 6 mg. per 100 cc. of blood.¹⁴¹ When the blood amino acid content is increased by injecting amino acids into the blood stream, there is a correlative increase in amino acid excretion.¹⁴² When proteins are fed in the form of, for instance, plant protein, in which the distribution is different from that in animals, the surplus amino acid over that utilized by the body is excreted.¹⁴¹ Amino acids act as precursors

of certain urinary excretory products; thus, arginine and histidine produce an increase in allantoin excretion, this substance being a CO_2 -less uric acid;¹⁴⁴ but while histidine can form purines like uric acid, it is not certain that arginine has this capacity.¹⁴⁵ Arginine does not increase creatinine excretion;^{145, 146} and the apparent increase in creatinine excretion after feeding this amino acid is due to the stimulation of metabolism which may be accomplished by administering other amino acids.^{147, 148} Glycine, on the other hand, especially in certain pathologic states,¹⁴⁹ leads to the excretion of more creatinine than before feeding glycine. Creatinine is not affected.¹⁵⁰ Phenylalanine gives rise to phenylacetic acid and small amounts of phenylpyruvic acid excretion in the urine; but 85 per cent of the amino acid fed is not recovered.¹⁵¹ Normally, tyrosine is excreted in a completely oxidized state as a phenol, but if this process is interrupted, homogentisic acid, or else *p*-hydroxyphenylpyruvic acid may be excreted; the former condition is known as *alkaptonuria*, the latter as *tyrosinosis*.¹⁵² Homogentisic acid, on a protein-free diet is excreted to the extent of about 2 Gm. per twenty-four hours.¹⁵³ This condition is caused by a defect in the oxidative procedure whereby a 2,5-position radical or group cannot be oxidized.

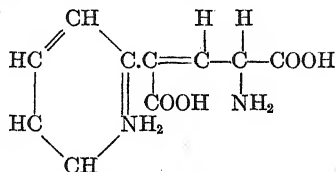
Other amino acids present special interest, those bearing aromatic rings (*e. g.*, phenylalanine) being excreted either as a phenyl-compound, or as a conjugation product with sulfuric acid. Histidine may be completely oxidized by opening the ring, but sometimes the oxidation is incomplete, formic acid being excreted.¹⁵³ Proline and hydroxyproline are not converted immediately into CO_2 , and H_2O and ammonia, for in the liver neither CO_2 , H_2O or NH_3 arises from such amino acids.¹⁵⁴ Cystine is excreted as CO_2 , H_2O and NH_3 and inorganic sulfate. In the disease known as *cystinuria*, the amino acid is excreted not in a free condition but in combination.¹⁵⁵ The disease, frequently referred to as an "inborn error of metabolism" is rare; out of 10,000 urines of college students, four showed marked, acute cystinuria.¹⁵⁶ When cystine is fed to such subjects, there is no increase in excreted cystine. Optically active cystine (and cysteine) in the dog is excreted about 70 per cent as oxidized sulfate and about 4 per cent as organic sulfur.⁶¹ Racemic cystine and cysteine are less liable to be oxidized, 16 per cent being left to be excreted as organic sulfur. Modification of the cystine by conjugation with acetyl-, chloracetyl-, or as glycyl-cystine leads to less oxidation, acetyl-cystine being excreted equally as sulfate and as organic sulfur. In the form of the tripeptide, glutathione, cystine excretion, however, resembles that of racemic cystine.

Hippuric acid, conjugated benzoic acid and glycine, represents the greatest quantity of urinary excretion of any amino acid. According to Quick,¹⁵⁷ glycine is constantly being synthesized in man to the extent of about 9 mg. per hour for each kilogram of body weight.

Imidazols, evidently derived from histidine, are excreted in amounts

of 150–600 mg. per twenty-four hours.¹⁵⁸ Protein administration leads to an increase in the excretion of such substances.

Tryptophan is excreted as phenol, conjugated with KHSO_4 in the form of indoxyl-potassium sulfate, or indican, but it appears also as kynurenin.¹⁵⁹ Experimentally, from about 4 Gm. of tryptophan fed, 3 Gm. (75 per cent) appears in the urine as kynurenin sulfate, the rest as kynurenic acid. Kynurenin is:



Kynurenin, probably, is the precursor of the urinary pigment, urochrome.¹⁵⁸

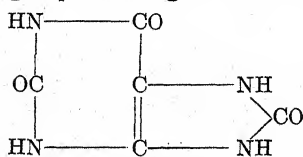
Finally, a third and perhaps important form of tryptophan excretion is indolepyruvic acid.

Phenylalanine, in addition to being excreted after cleavage of the ring, may also give rise to phenylpyruvic acid, homologous with indolepyruvic acid from tryptophan.

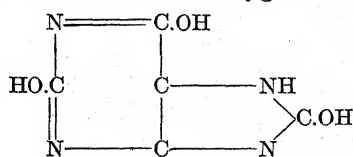
Only when a large amount of any amino acid is fed are abnormal symptoms detected.¹⁶⁰ There is reason to believe that after prolonged fasting, the feeding of glycine in liberal amounts is highly toxic.

Uric Acid.—Uric acid is excreted in amounts of about 0.7 Gm. per twenty-four hours. On a fasting diet, excretion varies from 25 mg. for the first three morning hours to 10 mg. in the evening; or from 0.3 to 0.5 Gm. per twenty-four hours of total excretion.¹⁶¹ Uric acid represents the final stage in the oxidation of purines and, to a certain extent, of methylpurines derived from plants.¹⁶² It occurs in urine as free uric acid in amounts of about 6.5 mg. per 100 cc. of urine. If an excess of salt (K , Na , NH_4) be present, then the solubility of uric acid is raised to 120 mg. per 100 cc. in which case we have urates. Actually, however, these concentrations in urine are not realized, owing to the action of Le Chatelier's law in suppressing the solubilities of the urates. According to Van Slyke's calculations⁴³ (p. 416, vol. I), the actual content must be of a magnitude of that of blood uric acid; namely, about 6 mg. per 100 cc. blood.

In stereochemical terms, uric acid, in solution, exists probably as mixture of two isomers, lactim and lactam,¹⁶³ the hydrogens of three groups shifting to a more stable position attached to an oxygen each:



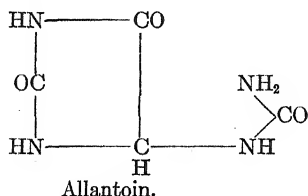
Lactim form of uric acid.



Lactam form of uric acid.

The acidity of uric acid is about fifty times that of carbonic acid.⁴³

Uric acid is derived from endogenous and from exogenous sources.¹⁶⁴ On a purine-free diet, the excretion is, of course, wholly endogenous. This does not mean, however, that all such uric acid is derived from nucleoproteins, for synthesis of uric acid may occur. In man, little uric acid is lost as allantoin, but in lower animals as



high as 98 per cent of metabolic uric acid is lost in this way. In fact, the loss of uric acid to allantoin in man and the higher anthropoid apes is not of metabolic origin, for it has been shown that the small content of allantoin in such urines is of extra, or exogenous origin.¹⁶⁵ How much destruction of uric acid occurs in the body and what the fate is of such a substance is not known. Older work showed a disappearance of about half the amount of uric acid experimentally administered, and this has been corroborated.^{166, 167} Wells' work¹⁶⁸ seems to relegate the *locus* of destruction of uric acid to the liver, although we must take into consideration the loss of some uric acid by excretion into the alimentary tract, where bacteria destroy it.¹⁶⁹ Allantoin is derived, in the dog, in part from the metabolism of extranuclear purines.¹⁷⁰ A uricolytic enzyme does not seem to be necessary for explaining the behavior of uric acid in the human body.

Animals, including man, are subject to certain abnormal forms of purine metabolism. Thus, the pig may suffer from guanidine gout. In man, gout is concerned with uric acid. Painful attacks of "rheumatism" occur at intervals, due to deposits of urates in cartilage and in muscles, setting up inflammation. On a purine-free diet, the patient excretes an amount of uric acid below the normal amount, until a crisis occurs, during which and immediately preceding it, the excretion of uric acid rises to twice the amount of the intercritical period. The modern picture of gout is that the kidney holds uric acid from passage into the urine until it can hold no more; then a flood of uric acid passes from the kidney into the urine.

Creatinine.—Of all the urinary products, it is the most characteristic and constant—so constant that many laboratories use it to indicate the completeness of urine collections.¹⁰⁹ Moreover, the constancy of creatinine excretion over short periods is maintained;¹⁷² for it is a variation in volume (water) that one observes in short-term studies of creatinine excretion rather than variations of creatinine excretion itself.¹⁷¹

Creatinine is excreted through the glomerulus and, by withdrawal of water through the tubule, concentrated to the urine content.¹⁹⁶

Shaffer proposed that "the amount of creatinine excreted depends primarily upon the mass of active protoplasmic tissues. The ratio: Milligrams creatinine nitrogen per kilo of body weight, I have called the *creatinine coefficient*.¹⁷² The normal coefficient ranges from about 7 to 11. Shaffer himself, 65 kilos body weight, spare and muscular, gave a coefficient of 9.

When creatinine is fed to experimental animals the creatinine of the urine does not increase. Thus, the creatinine excretion previous to administering creatinine was, per day, 0.251; 0.219; 0.135; 0.399 and 0.172; creatinine was fed and the first day gave 0.756 and later days 0.166; 0.318; 0.234; 0.694; 0.187. The apparent rise after 0.6 Gm. of creatinine of 0.756 Gm. is slight and is interpreted as due to the diuretic action of the creatinine creating a "wash-out" effect.¹⁷⁵

Creatinine is present in small amounts in infant's urine, but gradually it increases, reaching the adult excretion about the fourth or fifth year of life.¹⁷⁶

The creatinine coefficient of men is on the average 9 (5.4–11.7); for women, the average is lower, 6 being usually given (3.5–9.8).¹⁷² In women, there is more fat and muscles are less developed; but in athletic women, the coefficients are similar to those of men.¹⁷⁷

In fasting, creatinine excretion slowly falls. It may do so immediately, or it may occur only after three days. In any event, it remains fairly constant for the first week. Morgulis finds the following changes in creatinine during the fasting period of a dog:¹⁷⁸

| | Total creatinine | Preformed creatinine | Creatine |
|-------------------------------------|------------------|----------------------|----------|
| Preliminary period, three days..... | 0.332 | 0.125 | 0.197 |
| Fasting, four-day periods..... | .466 | .281 | .185 |
| | .432 | .137 | .295 |
| | .408 | .351 | .057 |
| | .442 | .349 | .093 |
| | .365 | .308 | .057 |
| | .355 | .283 | .072 |
| | .320 | .248 | .072 |
| | .168 | | .268 |
| | .153 | | .280 |

In the last two periods, the excretion of creatine is higher than that of creatinine. If these quantities were plotted, the two curves, that of creatinine and creatine would cross. This is the "creatine crossing" of Hawk¹⁷⁹ which Morgulis¹⁷⁸ does not believe of significance as indicating the onset of death by starvation, as the original writers¹⁷⁹ believed. The creatine is not due to supersaturation of the muscles with creatine but, doubtless, to an alteration in the metabolism of sugar.¹⁸⁰

If, in place of starvation, a protein-free diet be fed, creatinine is excreted uniformly.¹⁸¹ Meat fed in copious amounts (200 Gm. per day) does not affect creatinine excretion; in fact, the feeding of pure creatine is without effect on the creatinine excretion.

When neither creatine nor creatinine is present in the food, excreted creatinine is proportional to muscle mass.^{182, 183} We have

spoken of the effect in women of muscle development on creatinine excretion; as the musculature increases in efficiency and bulk, the creatinine excretion tends to approach that of the male.

During sleep, the tonus of muscle is maintained, yet work is practically eliminated. In such a condition and in prolonged rest in bed, creatinine excretion is said to be lessened,¹⁸⁴ but Simpson¹⁷⁴ tried to connect it with water retention under such states as sleep and rest. Immediately at the beginning of a working period, there is a slight rise in creatinine excretion, which continues to a maximum in about one hour.¹⁸⁵ As to "tonus," there is little unanimity of opinion. The older conception of Pekelharing¹⁸⁶ was criticized by Schulz,¹⁸⁷ who found no relation between tonus and creatinine excretion.

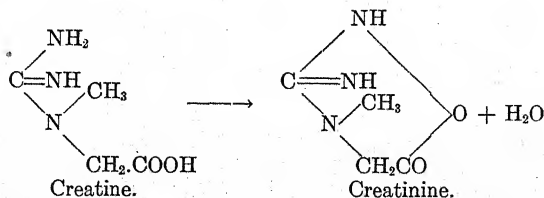
Menstruation does not seem to affect the excretion of creatinine.^{188, 189} The same may be said of menopause and pregnancy.

The speed of excretion may be realized from the fact that hypernormal concentration of creatinine in blood leads to 147 per cent clearance over that of urea.¹⁹⁷

Creatine.—From the ages of birth to near puberty, creatine is excreted by both sexes, later in the female than in the male, although it is probable that the differences in sex depend upon muscle development.¹⁹⁰ Thereafter (about the sixteenth year), creatine is abnormal in male urine, but it is characteristic of female urine during sexual events.¹⁹¹ Modern views favor the idea that creatinuria in the female is largely concerned with the lower development of muscle and fails as the musculature is brought nearer that of the male; or as Hunter¹⁷⁶ expresses it, "creatinuria of women represents merely an imperfect transition from the creatine metabolism of childhood." Creatinuria of parturition, however, is well recognized;¹⁹¹ the intermittent creatinuria of the female becomes a continuous one. Postpartum urine contains a high concentration of creatine.¹⁷² As creatine increases creatinine excretion becomes less.¹⁹⁰

By hysterectomy, Mellanby¹⁹² proved that creatinuria was not due to the involution of the gravid uterus, but rather is somehow connected with lactation; but how the activity of the mammary glands is correlated with creatine production or excretion, we do not know.

Although chemical differences between creatine (methylguanidine acetic acid) and its anhydride, creatinine involve only a molecule of water:



yet the proof of the origin of urinary creatinine from muscle or blood creatine is not available. The creatinine of blood is known to be identical with urinary creatinine.¹⁹³ When creatine is administered

to dogs over an extended period of five to ten weeks, a gradual increase of excreted creatinine is found.¹⁹⁴ In the tenth week of one such case, the output was one-third more than the original level. Only after ten days did creatine occur in the urine and remained constant throughout the remainder of the experiment. Cessation of creatine administration was followed by disappearance of creatine from the urine. The slowness of this conversion of creatine to creatinine indicates a complex series of intermediate chemical transformations, although what these are is wholly unknown.

In man, creatine in the urine appears during the third day of fasting. The origin of this creatine is obscure, but two hypotheses have been offered: (a) Muscle, during fasting, loses its tone and consequently its creatine; (b) impotence of the body to dehydrate creatine to form creatinine. Brand and Harris¹⁹⁵ involve glycine in creatine formation, or perhaps glutathione, which has glycine in its molecule. It is conceivable that fasting creatinuria is due to unusual metabolism of glycine or glutathione.²⁰¹

The Total Nitrogen of the Urine.—The sum of the various nitrogenous substances excreted in the urine is known as "total nitrogen." The term "nonprotein nitrogen" refers only to pathologic urines in which albumin occurs. It was shown in the Munich Physiological Institute years ago that all metabolic nitrogen passed off in the urine, a negligible portion being lost in the feces, perspiration and epidermal substances like hair and cuticle. Fecal nitrogen remains constant at about 0.1 per cent of the nitrogen intake, but it persists even on a protein-free diet.¹⁹⁸ Such nitrogen is largely that derived from the secretions of the alimentary tract, which is increased on feeding substances like potatoes that increase the bulk of the feces.¹⁹⁹ Such nitrogen seems to be about 0.2 Gm. per 100 Gm. of substance or 5 Gm. per day; when roughage is not given, it falls to 1-2 Gm. per twenty-four hours.

Nitrogen balance indicates that approximately the same amount of nitrogen ingested is excreted. This varies with the individual, but approximates to about 12 Gm. per twenty-four hours. In fasting, the total nitrogen falls to 7 Gm., and even when food is resumed, a further fall to as low as 4 Gm. per twenty-four hours may occur, due to the specific dynamic action of the foods. Nitrogen balance may be established at any level of protein intake beyond about 0.6 Gm. of protein per kilo of body weight. Below that amount, equilibrium cannot be established. The character of the protein ingested is another factor. Incomplete proteins like gelatin cannot be used to obtain nitrogenous equilibrium, owing to its deficiency in certain amino acids.

"Undetermined" Nitrogen of the Urine.—When the sum of the known constituents of the urine is subtracted from total nitrogen, there may be a residual quantity called "undetermined" nitrogen. The character of this quantity is unknown, but in some cases, amines (like trimethylamine) have been identified.

Variations in Urine Volume.—*Anuria* is complete suppression of urinary excretion. It occurs physiologically in cases of profuse sweating over short periods, but wholly in a transitory way. Under pathologic conditions, especially in mercury poisoning, anuria is a characteristic phenomenon. *Polyuria* is a condition of excess urinary volume, encountered physiologically in high protein diets, due to the diuretic effects of urea; or in the ingestion of beverages (coffee and tea, containing the methylpurines caffeine and theophylline). *Oliguria* is suppressed urine excretion, observed in low protein diets, or the usual profuse perspiration of warm weather.

Reaction of Urine.—The average normal hydrogen ion concentration expressed as pH is 6.²⁰⁰ At that degree of acidity, litmus is blue. This acidity is the equilibrium established by the kidney in regulating the acid-base relations in the body, which may vary with food, environment and other factors. While the lungs excrete CO_2 and H_2O exclusively, the kidney deals with nonvolatile substances such as carbonates, phosphates, etc. The carbonate is a function of the CO_2 tension of the urine which approximates that of the blood, namely, about 40 mm. of mercury. Besides CO_2 , the kidney is concerned with other acids, of which the phosphate is very important. At pH 8.2 bicarbonate is converted into carbonate and this pH is the maximum attainable by the kidney in normal states. Bases are excreted in equilibrium with those of the blood. In the usual reaction of the urine, 87 per cent of the phosphate is in the form of the acid salt, NaH_2PO_4 . This is the characteristic acid-producing substance of the urine.

The term "total urinary acidity" is sometimes used. It means the "titratable acidity" plus ammonia, in which the total available replaceable hydrogen plus that represented by ammonia as a neutralizing agent are the factors.

During digestion of food, the stomach secretes hydrochloric acid and chlorion is lost to the body. Consequently about an hour after a meal, especially one containing proteins, the urine becomes alkaline and the condition is known as the "alkaline tide," due to the augmented excretion of base which accompanies the increase in CO_2 -tension. At this time, ammonia is reduced owing to the excretion of base.

In fasting, fats are incompletely burned and organic acids like acetoacetic acid are excreted. This calls upon the alkali reserve, the depletion of which is called "acidosis." *Anoxemia* (oxygen want) causes acidosis, which, if prolonged, produces a reduction of urinary pH , but for brief periods, there is a "blowing-off" of CO_2 and an alkalosis may result, such as follows climbing at high altitudes. The effect of *muscle work* varies with the previous condition of the subject and with the degree of exercise. Anoxemia may exist if the gaseous exchange is interrupted. There is a rapid excretion of acid in the urine, consisting of phosphate and ammonia.

Acids of Cl , SO_4 , etc., reduce the amount of alkali in blood and

urine. As salts (NaCl) their effect is less. Na and K salts of organic acids that are burned in the body leave an excess of Na or K which is excreted as carbonates. Other salts are acid-producing. Thus, CaCl_2 , CaSO_4 and $\text{Ca}(\text{NO}_3)_2$ are metabolized as acids because the calcium is either unabsorbed from the intestine, or is excreted in large part through that organ, leaving the acid radicals free to become effective as acids.

WITHROW MORSE.

REFERENCES

1. Freeman, B., Livingston, A. E., and Richards, A. N.: *J. Biol. Chem.*, **87**, 467 (1930).
2. Richards, A. N., and Walker, A. M.: *J. Biol. Chem.*, **87**, 479 (1930).
3. Walker, A. M.: *J. Biol. Chem.*, **87**, 499 (1930).
4. Bayliss, L. E., and Walker, A. M.: *J. Biol. Chem.*, **87**, 523 (1930).
5. Walker, A. M., and Elsom, K. A.: *J. Biol. Chem.*, **91**, 593 (1931).
6. Wearn, J. T., and Richards, A. N.: *Am. J. Physiol.*, **71**, 209 (1924).
7. Ekehorn, G.: *Acta med. Scan.* (Suppl.) (1931).
8. White, H. L.: *Am. J. Physiol.*, **85**, 191 (1928).
9. Ludwig, C.: *Handbuch der Physiologie des Menschen*. See Cushny's *Secretion of the Urine* (1925).
10. McIntosh, R., Kajdi, L., and Meeker, D.: *J. Clin. Invest.*, **9**, 333 (1930).
11. Swanson, P. P., and Smith, A. H.: *J. Biol. Chem.*, **98**, 479 (1932).
12. Simone, I.: *Biochim. terapia sperim.*, **17**, 469 (1930).
13. Rabbeno, A.: *Arch. ital. Biol.*, **83**, 73 (1930).
14. Hubbard, R. S., Munford, S. A., Tynner, J., and Allison, C. B.: *J. Biol. Chem.*, **92**, xxix (Proc.) (1931).
15. Mosher: *J. Biol. Chem.*, **99**, 781 (1931).
16. Hastings, A. B., Harkins, H. N., and Liu, S. K.: *J. Biol. Chem.*, **94**, 681 (1932).
17. McLean, F. C.: *J. Exp. Med.*, **22**, 212 and 366 (1915).
18. Ambard, L.: *Physiologie normal et pathol. des reins* (1920).
19. Marshall, E. K.: *J. Pharmacol.*, **16**, 141 (1920).
20. Ambard, L., et Schmid, F.: *Bull. et Mem. Soc. med. hop. Paris*, **51**, 82 (1927).
21. Cameron, A. T., and Hollenberg, M. S.: *J. Biol. Chem.*, **44**, 239 (1920).
22. Hanke, M. E., and Donovan, P. V.: *Proc. Soc. Exptl. Biol. Med.*, **24**, 580 (1927).
23. Whelan, M.: *J. Biol. Chem.*, **63**, 585 (1925).
24. Talbert, G. A., and Haugen, C. O.: *Am. J. Physiol.*, **81**, 74 (1927).
25. Hastings, A. B., and van Dyke, H. B.: *J. Biol. Chem.*, **78** (Proc.), xxxv (1928).
26. Baird, M. M., and Haldane, J. B. S.: *J. Physiol.*, **56**, 259 (1922).
27. Wesselow, de, O. L. V.: *Quart. J. Med.*, **19**, 53 (1925).
28. Salkowski, E.: *Arch. f. Anat. Physiol.*, **53**, 209 (1871).
29. Kramer, B., and Tisdall, F. F.: *J. Biol. Chem.*, **53**, 241 (1922).
30. Schloss, E.: *Z. Kinderheilk.*, **3**, 441 (1912).
31. Magnus-Levy, A.: *Deut. med. Wochschr.*, **46**, 594 (1920).
32. Leiter, L.: *J. Clin. Invest.*, **3**, 253 (1926).
33. Miller, H. G.: *J. Biol. Chem.*, **67**, 71; **70**, 587, 593 (1926).
34. Gamble, J. L., Blackfan, K. D., and Hamilton, B.: *J. Clin. Invest.*, **1**, 359 (1925).
35. Moss, K. N.: *Proc. Roy. Soc. (London)*, **95B**, 181 (1923).
36. Wiley, F. H., Wiley, L. L., and Waller, D. S.: *J. Biol. Chem.*, **97** (Proc.), lvi (1932).
37. Shohl, A. T.: *Physiol. Rev.*, **3**, 509 (1923).
38. Givens, M. H., and Mendel, L. B.: *J. Biol. Chem.*, **31**, 421 (1917).
39. Zucker, T. F.: *Proc. Soc. Exp. Biol. Med.*, **18**, 272 (1921).
40. Watchorn, E.: *Quart. J. Med.*, **18**, 288 (1925).
41. Euler, H. von, Nilsson, R., and Auhagen, E.: *Z. physiol. Chem.*, **200**, 1 (1931).
42. Kruse, H. D., Orent, E. R., and McCollum, E. V.: *J. Biol. Chem.*, **100** 603 (1933).

43. Peters, J. P., and Van Slyke, D. D.: *Quantitative Clinical Chemistry* (1931).
44. Sawyer, M., Baumann, L., and Stevens, F.: *J. Biol. Chem.*, **33**, 103 (1918).
45. Wellmann, O.: *Arch. ges. Physiol.*, **121**, 508 (1907).
46. Mathison, G. C.: *Biochem. J.*, **4**, 274 (1908).
47. Henderson, L. J.: *The Fitness of the Environment* (1914).
48. Lohmann, K.: *Biochem. Z.*, **203**, 172 (1928).
49. Addis, T., Meyers, B. A., and Bayer, L.: *Am. J. Physiol.*, **72**, 125 (1925).
50. Haldane, J. B. S., Wigglesworth, V. B., and Woodrow, C. E.: *Proc. Roy. Soc. (London)*, **96B**, 15 (1924).
51. Stehle, C. L., and McCarty, A. C.: *J. Biol. Chem.*, **47**, 315 (1921).
52. Lamb, A. R., and Evvard, J. M.: *J. Biol. Chem.*, **37**, 329 (1919).
53. Brull, L., and Eichholtz, F.: *Proc. Roy. Soc. (London)*, **99B**, 70 (1925).
54. Kay, H. D.: *Biochem. J.*, **20**, 791 (1926).
55. Brain, R. T., Kay, H. D., and Marshall, P. G.: *Biochem. J.*, **22**, 628 (1928).
56. Walker, A. M.: *J. Biol. Chem.*, **101**, 239 (1933).
57. Schmitt, F. O., and White, H. L.: *Am. J. Physiol.*, **84**, 401 (1928).
58. Robison, R.: *Biochem. J.*, **17**, 286 (1923).
59. Bollinger, A.: *J. Biol. Chem.*, **76**, 797 (1928).
60. Hendrix, B. M., and Calvin, D. B.: *J. Biol. Chem.*, **65**, 197 (1925).
61. Hele, T. S., and Pirie, N. W.: *Biochem. J.*, **25**, 1095 (1931).
62. Brand, E., Harris, M. M., and Biloon, S.: *J. Biol. Chem.*, **86**, 315 (1930).
63. Delepine, S.: *Proc. Roy. Soc. (London)*, **47B**, 198 (1890).
64. Drabkin, D. L.: *J. Biol. Chem.*, **67**, xl (Proc.) (1926).
65. Looney, J. M., Berglund, H., and Graves, R. C.: *J. Biol. Chem.*, **57**, 515 (1923).
66. Schmidt, C. L. A., and Allen, E. G.: *J. Biol. Chem.*, **42**, 55 (1920).
67. Sullivan, M. X., and Dawson, P. R.: *J. Biol. Chem.*, **45**, 473 (1920).
68. Abel, J. J., and Geiling, E. M. K.: *J. Pharmacol.*, **24**, 423 (1925).
69. Hele, T. S.: *Biochem. J.*, **18**, 110 (1924).
70. Du Vigneau, V., Jensen, H., and Wintersteiner, O.: *J. Pharmacol.*, **32**, 367 (1927).
71. Pelkan, K. F.: *J. Biol. Chem.*, **50**, 491 (1922).
72. Denis, W., and Reed, L.: *J. Biol. Chem.*, **73**, 51 (1927).
73. Hele, T. S.: *Biochem. J.*, **25**, 1736 (1931).
74. Schelling, V.: *Am. J. Physiol.*, **102**, 714 (1932).
75. Folin, O.: *Am. J. Physiol.*, **13**, 45 (1905).
76. Cuthbertson, D. P.: *Biochem. J.*, **25**, 236 (1931).
77. Benedict, S. R.: *Carnegie Inst. Wash. Pub.*, No. 77 (1907).
78. Cathcart, E. P., and Burnett, S.: *Proc. Roy. Soc. (London)*, **99B**, 159 (1911).
79. Cuthbertson, D. P.: *Biochem. J.*, **1328** (1929).
80. Voit, C.: *Hermann's Handb. der Physiol.* (1881).
81. Wilson, H. E. C.: *J. Physiol.*, **72**, 327 (1931).
82. Wilson, H. E. C.: *Biochem. J.*, **19**, 322 (1925); **20**, 76 (1926).
83. Carpenter, F. M.: *J. Biol. Chem.*, **55**, iii (Proc.) (1923).
84. Dutoit, P., et Zbniden, C.: *Compt. rend.*, **190**, 172 (1930).
85. Rose, M. S.: *Yale J. Biol.*, **4**, 499 (1932).
86. Underhill, F. P., and Peterman, F. I.: *Am. J. Physiol.*, **90**, 1 (1929).
87. Mackenzie, K.: *Biochem. J.*, **24**, 1433 (1930).
88. Fairhall, L. T.: *J. Biol. Chem.*, **70**, 495 (1926).
89. Fox, H. R., and Ramage, H.: *Proc. Roy. Soc. (London)*, **108B**, 157 (1931).
90. Reiman, C. K., and Minot, A. S.: *J. Biol. Chem.*, **42**, 329 (1920).
91. Austin, J. H., Stillman, E., and Van Slyke, D. D.: *J. Biol. Chem.*, **46**, 91 (1921).
92. Moeller, E., McIntosh, J. F., and Van Slyke, D. D.: *J. Clin. Invest.*, **6**, 427 (1928).
93. Rouelle, F. M.: *Journ. de Med.*, November, 1773.
94. Prout, W.: *Ann. chim.* (2), **10**, 369 (1817).
95. Hopkins, F. G.: *Biochem. J.*, **22**, 1341 (1928).
96. Woehler, F.: *Ann.*, **12**, 243 (1928).
97. Werner, E. A.: *Urea* (1923).
98. Dumas, J. B.: *Ann. chim.*, **2**, 44 (1830).
99. Chattaway, F. D.: *Trans. Chem. Soc.*, **101**, 170 (1912).

100. Devato, G.: *Gazz. chim. ital.*, **65**, 520 (1930).
101. Arndt, L.: *Ber.*, **63**, 2963 (1930).
102. Ebert, L.: *Ber.*, **64**, 679 (1931).
103. Werner, E. A.: *Trans. Chem., Soc.* **111**, 863 (1917).
104. Krebs, H. A., und Henseleit, K.: *Klin. Wochschr.*, **11**, 757 (1932).
105. Koga, Y., and Otake, S.: *J. Chem. Soc.*, Tokio, **35**, 519 (1914).
106. Bollman, J. L., Mann, F. C., and Magath, T. B.: *Am. J. Physiol.*, **69**, 371 (1924).
107. Holmes, B. E., and Watchorn, E.: *Biochem. J.*, **21**, 327 (1927).
108. Edlbacher, S., und Bonem, P.: *Z. physiol. Chem.*, **145**, 69 (1925).
109. Folin, O.: *J. Am. Med. Assoc.*, **69**, 1209 (1917).
110. Lennox, W. G., O'Connor, M., and Bellinger, M.: *Arch. Internal Med.*, **38**, 355 (1926).
111. Moore, D. D., Laviates, P. H., Wakeman, A. M., and Peters, J. P.: *J. Biol. Chem.*, **91**, 373 (1931).
112. Pruefer, J.: *Z. klin. Med.*, **114**, 293 (1930).
113. Kiech, V. C., and Luck, J. M.: *J. Biol. Chem.*, **94**, 433 (1931).
114. Salaskin, S., and Solowjew, L.: *Z. physiol. Chem.*, **192**, 28 (1930).
115. Kase, K.: *Biochem. Z.*, **233**, 271 (1931).
116. McQuarrie, I., and Whipple, G. H.: *J. Exp. Med.*, **29**, 397 (1919).
117. Maclean, H., and de Wesselow, O. L. V.: *Brit. J. Exp. Path.*, **1**, 53 (1920).
118. Ambard, L., and Weill, A.: *J. Physiologie et Path. génér.*, **14**, 753 (1912).
119. Jacoby, M.: *Biochem. Z.*, **84**, 354 (1917).
120. Takeuchi, Y., and Jonone, K.: *J. College Agr., Imp. Univ. Tokio*, **1**, 1 (1909).
121. Armstrong, H. E., and Horton, E.: *Proc. Roy. Soc. (London)*, **85B**, 109 (1912).
122. Fearon, W. R.: *Biochem. J.*, **17**, 84 (1923).
123. Waldschmidt-Leitz, E.: *Enzyme Actions* (1929).
124. Fasse, R.: *Compt. rend.*, **145**, 813 (1907).
125. Luck, J. M., and Seth, T. N.: *Biochem. J.*, **19**, 357 (1925).
126. Benedict, S. R., and Nash, T. P., Jr.: *J. Biol. Chem.*, **82**, 673 (1929).
127. Nash, T. P., Jr., and Benedict, S. R.: *J. Biol. Chem.*, **48**, 463 (1921).
128. Nash, T. P., Jr., and Williams, E. F., Jr.: *J. Biol. Chem.*, **94**, 783 (1932).
129. Wassermeyer, H.: *Arch. exp. Path. Pharmacol.*, **165**, 420 (1932).
130. Hasselbalch, K. A.: *Biochem. Z.*, **74**, 18 (1916).
131. Mainzer, F.: *Klin. Wochschr.*, **6**, 1689 (1927).
132. Musella, M.: *Klin. Wochschr.*, **11**, 1354 (1932).
133. Ducco, C. L.: *Arch. med. enf.*, **35**, 12 (1932).
134. Bornstein, A.: *Biochem. Z.*, **212**, 137 (1929); **214**, 374 (1929).
135. Kohn, R.: *Z. physiol. Chem.*, **200**, 191 (1931).
136. Bollman, J. L., and Mann, F. C.: *Am. J. Physiol.*, **92**, 92 (1930).
137. Embden, G., and Deutlicke, H. J.: *Z. physiol. Chem.*, **190**, 62 (1930).
138. Holmes, B. E., and Patey, A.: *Biochem. J.*, **23**, 760 (1929).
139. Rigoni, M.: *Arch. ital. biol.*, **84**, 74 (1931).
140. Van Slyke, D. D., Linder, G. C., Hiller, A., Leiter, L., and McIntosh, J. F.: *J. Clin. Invest.*, **2**, 255 (1926).
141. Schmitz, E., and Simon, P.: *Biochem. Z.*, **160**, 1 (1925).
142. Van Slyke, D. D., and Meyer, G. M.: *J. Biol. Chem.*, **16**, 213 (1913).
143. Levene, P. A., and Kober, P. A.: *Am. J. Physiol.*, **23**, 324 (1908).
144. Ackroyd, H., and Hopkins, F. G.: *Biochem. J.*, **10**, 551 (1916).
145. Rose, W. C., and Cook, K. G.: *J. Biol. Chem.*, **64**, 325 (1925).
146. Thomas, K.: *Z. physiol. Chem.*, **88**, 465 (1913); **92**, 163 (1914).
147. Gross, E. G., and Steenbock, H.: *J. Biol. Chem.*, **47**, 35 (1921).
148. Grant, R. L., Christman, A. A., and Lewis, H. B.: *Proc. Soc. Exp. Biol. Med.*, **27**, 231 (1929).
149. Brand, E., Harris, M. M., Sandberg, M., and Lasker, M. M.: *J. Biol. Chem.*, **87** (Proc.), ix (1930); *Amer. J. Physiol.*, **90**, 296 (1929).
150. Zwarenstein, H.: *Biochem. J.*, **22**, 307 (1928).
151. Chandler, J. P., and Lewis, H. B.: *J. Biol. Chem.*, **96**, 619 (1932).
152. Medes, G.: *Biochem. J.*, **26**, 917 (1932).
153. Zeyen, M.: *Z. klin. Med.*, **120**, 128 (1932).
154. Bernheim, F., and Hernheim, M. L. C.: *J. Biol. Chem.*, **96**, 325 (1932).
155. See No. 62.
156. Lewis, H. B.: *Annals of Internal Medicine*, **6**, 183 (1932).

157. Quick, A. J.: *J. Biol. Chem.*, **92**, 65 (1931).
158. Kaufmann, F., und Engel, R.: *Z. klin. Med.*, **114**, 405 (1930).
159. Matsuoka, Z., und Yoshimatsu, N.: *Z. physiol. Chem.*, **143**, 206 (1925).
160. Newburg, L. H., and Marsh, P. L.: *Arch. Internal Med.*, **36**, 682 (1925).
161. Neuwirth, I.: *J. Biol. Chem.*, **29**, 477 (1917).
162. Myers, V. C., and Wardell, E. L.: *J. Biol. Chem.*, **77**, 697 (1928).
163. Baeyer, H. von: *Ber.*, **15**, 2093 (1882).
164. Siven, V. O.: *Skand. Arch. Physiol.*, **11**, 123 (1901).
165. Ackroyd, H.: *Biochem. J.*, **5**, 400 (1909).
166. Burian, R., und Schur, H.: *Arch. ges. Physiol.*, **87**, 239 (1910).
167. Folin, O., Berglund, H., and Derick, C.: *J. Biol. Chem.*, **60**, 361 (1924).
168. Wells, H. G.: *J. Biol. Chem.*, **26**, 319 (1916).
169. Luck, H.: *Z. ges. exper. Med.*, **70**, 488 (1930).
170. Allen, F. W., and Cerecedo, L. R.: *Proc. Soc. Exptl. Biol. Med.*, **29**, 190 (1931).
171. Simpson, G. E.: *J. Biol. Chem.*, **59**, 107 (1924).
172. Shaffer, P. A.: *Am. J. Physiol.*, **23**, 1 (1908).
173. Folin, O.: *Z. physiol. Chem.*, **41**, 223 (1904).
174. See No. 171.
175. Foster, N. B., and Fisher, H. L.: *J. Biol. Chem.*, **9**, 359 (1911).
176. Hunter, A.: *Creatine and Creatinine* (1928).
177. Tracy, M., and Clark, E. E.: *J. Biol. Chem.*, **19**, 115 (1914).
178. Morgulis, S.: *J. Biol. Chem.*, **83**, 299 (1929).
179. Howe, P. E., Mattill, H. A., Hawk, P. B.: *J. Biol. Chem.*, **10**, 417 (1911).
180. Chanutin, A., and Silvette, H.: *J. Biol. Chem.*, **80**, 589 (1928).
181. Deuel, H. J., Sandiford, I. S., Sandiford, K., and Boothby, W. M.: *J. Biol. Chem.*, **76**, 391 (1928).
182. McClugage, H. B., Booth, G., and Evans, F. A.: *Am. J. Med. Sci.*, **181**, 349 (1931).
183. Hodgson, P., and Lewis, H. B.: *Am. J. Physiol.*, **87**, 288 (1928).
184. Eimer, K.: *Z. ges. exper. Med.*, **75**, 428 (1931).
185. Kael, K.: *Biochem. Z.*, **245**, 452 (1932).
186. Pekelharing, C. A.: *Z. physiol. Chem.*, **75**, 207 (1911).
187. Schulz, W.: *Arch. ges. Physiol.*, **186**, 126 (1921).
188. Wang, C. C., and Dentler, M. L.: *J. Biol. Chem.*, **45**, 237 (1920).
189. Okey, R.: *J. Biol. Chem.*, **63** (Proc.), xxxiii (1925).
190. Harding, V. J., and Gaebler, O. H.: *J. Biol. Chem.*, **54**, 579 (1922).
191. Krause, R. A., and Cramer, W.: *J. Physiol.*, **40** (Proc.), lxi (1910).
192. Mellanby, E.: *Proc. Roy. Soc. (London)*, **86B**, 88 (1913).
193. Gaebler, O. H., and Keltch, A. K.: *J. Biol. Chem.*, **74** (Proc.), xx (1927).
194. Benedict, S. R., and Osterberg, E.: *J. Biol. Chem.*, **56**, 229 (1923).
195. Brand, E., and Harris, M. M.: *Science*, **77**, 589 (1933).
196. Descombes, E.: *Biochem. Z.*, **246**, 59 (1932).
197. Jolliffe, N., and Smith, H. W.: *Am. J. Physiol.*, **98**, 572 (1931).
198. Smith, M.: *J. Biol. Chem.*, **68**, 15 (1926).
199. Mitchell, H. H.: *Bull. Nat. Research Council*, **1**, 21 (1926).
200. Henderson, L. J., and Palmer, W. W.: *J. Biol. Chem.*, **17**, 305 (1914).
201. Tripoli, C. J., and Beard, H. H.: *Arch. Internal Med.*, **53**, 435 (1934).

CHAPTER XXIX

THE BIOCHEMISTRY OF THE BRAIN

THE biochemistry of the central nervous system has received but scant attention until recent years. Possibly this is true because the brain has been surrounded, in the minds of men, with an aura which suggested certain vague philosophical conceptions concerning the "body-mind" problem. Whatever its cause, it was not until 1884, when J. L. W. Thudichum published his *Physiological Chemistry of the Brain*, that recognition was given to the fact that the brain might have any metabolism. Following Thudichum's death interest again lagged, to be temporarily revived by Waldemar Koch, but again to fall to a low ebb. Recently, investigations have been resumed in the hope that some light might be thrown on the great and pressing problem of mental and nervous diseases. Up to the present time, there is but little firm ground, established by the arduous labor of investigating the normal constituents and metabolism of brain tissue, on which excursions into the pathologic domain may be based. This chapter therefore proposes to sketch in the barest outline only certain more or less established facts about the chemistry of the brain without attempting to offer interpretations which, in the present state of our knowledge, would be premature and only serve to obscure further already obscure problems.

CARBOHYDRATE METABOLISM OF BRAIN

The brain contains glycogen but in relatively small amounts, and that which is present does not appear to be easily mobilized as is the case with other organs. It is questionable whether during the ordinary course of metabolism it is utilized. Comparison may be made between the glycogen of the brain and that deposited in spleen and liver during the course of the so-called "glycogen disease" (v. Gierke) or that in the kidneys of diabetic patients.

Inosite (hexahydroxycyclohexane) occurs in the brain in relatively large quantities. Probably it is synthesized by the body. Nothing is known of its function in the metabolism of nerve tissue.

Reducing substances occur in brain in small quantities, of which about 20 per cent is pentose. Much of the remainder is creatine and creatinine which reduce the Hagedorn-Jensen sugar reagent, in this way yielding false analytical values for "sugar" in cerebral tissue.

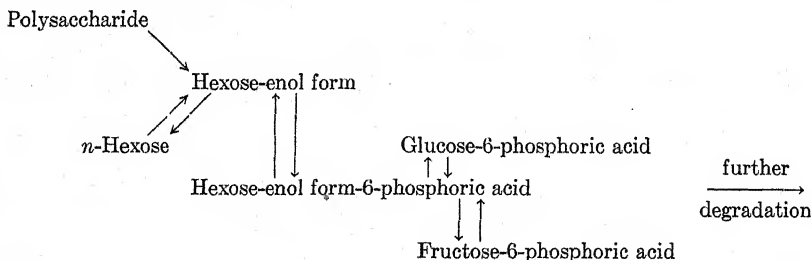
The amount of glucose appears to depend on the amount contained in the blood.¹ It is probable that no other organ is so immediately dependent on the sugar of the blood as brain. This fact suggests the close relationship of central nervous system symptoms and signs following overdosage with insulin.

Galactose apparently occurs only in combination as cerebroside. Pentose sugars, such as ribose, are found in the nucleotides.

Substances which are probably carbohydrate intermediaries have been isolated from fresh brain. Among them may be mentioned lactic acid, hexosemonophosphoric acid, methyl glyoxal, succinic acid, acetaldehyde, ethyl alcohol, glycerophosphoric acid and possibly pyruvic acid.

Much of the information regarding the metabolism of sugar in brain is derived by analogy from the mechanism in muscle. Since the latter is but incompletely understood, and in a rapid state of flux, data concerned with brain tissue are ordinarily well out of date when published. The phenomenon of glycolysis has furnished a large amount of information, which when applied to nervous tissue will surely yield results of great importance. It is for these reasons that we must briefly sketch work which has not as yet been shown to be directly applicable to cerebral tissue.

General Nature of Carbohydrate Degradation in Brain.*—The brain absorbs both glucose and lactic acid from the blood stream. It is possible that preliminary phosphorylation is necessary before this may take place. Esterification with phosphoric acid to form hexosemonophosphoric acid seems to be the first step in the breakdown of glucose or fructose. It is believed that the glucose-6-phosphoric acid so formed rearranges over the enol form into fructose-6-phosphoric acid. When isolated, the enol form stabilizes itself as the Robison ester; *i. e.*, as a mixture of ketose and aldose components:

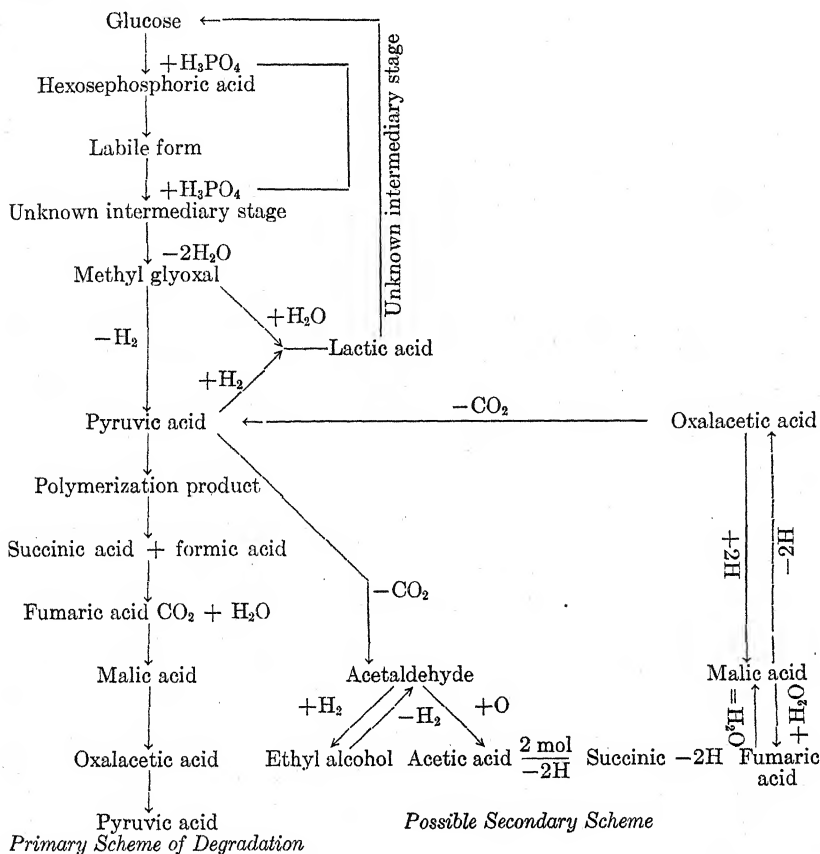


Splitting of the 6-carbon atom hexosephosphoric acid probably proceeds to the 3-carbon atom stage by virtue of the action of enzyme activated by coenzyme.

Certain investigators believe that methyl glyoxal is the first product of this splitting of ester. Oxidation begins at the methyl glyoxal stage with formation of pyruvic acid.² Much of the methyl glyoxal is not oxidized because it is converted into lactic acid by the action of glyoxalase. The lactic acid so formed may be resynthesized to glycogen or may be further degraded. At least, in muscle, pyruvic acid is probably converted in part into succinic acid, and the latter in the presence of hydrogen acceptor is transformed into fumaric acid. The

* In this connection, the reader might well refer to Chapters XX and XXVI.

further decomposition need not concern us here. We may schematize the above data as follows:



Quite recently Embden³ has proposed another scheme which appears to fit certain observed facts better than the possibilities just described. Essentially, Embden stresses the importance of the production of glyceric-aldehyde phosphoric acid, α -glycerophosphoric acid and pyruvic acid in intermediary carbohydrate metabolism:

- | | |
|--|--|
| A. Fructose diphosphoric acid | = 1 Glyceric-aldehyde phosphoric acid + 1 dioxycetone phosphoric acid. |
| B. 1 Glyceric-aldehyde phosphoric acid + 1 dioxycetone phosphoric acid | = 1 α -glycerophosphoric acid + 1 phosphoglyceric acid. |
| C. 1 Phosphoglyceric acid | = 1 Pyruvic acid + 1 phosphoric acid |
| D. 1 Pyruvic acid + 1 α -glycerophosphoric acid | = 1 Lactic acid + 1 triosephosphoric acid. |

Whether this hypothesis will be supported by future investigations cannot at present be foretold⁴ but evidence is rapidly accumulating which lends to substantiate it.

Glycolysis in Brain.—Brain tissue behaves, as regards its glycolytic activity—that is to say, its power to cause the disappearance of glucose—in many respects like neoplastic tissue; thus being sharply separated from other “normal adult tissues.”⁵ Gray matter, like neoplastic tissue, has a high glycolysis under both aerobic and anaerobic conditions, and also a relatively low respiration rate. In normal adult tissues, glycolysis is reduced by the admission of oxygen (Pasteur reaction) and, furthermore, exhibits a high rate of oxygen consumption (*i. e.*, respiration).

Various substances influence the rate of glycolysis in brain to a considerable extent. *l*-Lactate inhibits glycolysis to about one half that of *d*-lactate. Inhibition of the enzyme reaction by addition of an end-product, such as lactate, is probably due to a combination of added substance with the enzyme system.

Lactic acid is produced by brain tissue *in vitro* in the presence of glucose with great rapidity.

The dependence of glycolysis in cerebral tissue on glucose is well illustrated by the experiments of Dickens and Greville.⁶ A period of anaerobic substrate (ordinarily glucose) deprivation spontaneously decreases the ability of the tissue subsequently to glycolyze added glucose. Other tissues under the same treatment recover this function almost completely. Absence of glucose has less effect on subsequent respiration in glucose than on glycolysis.

Sodium pyruvate in low concentration causes acceleration of anaerobic glycolysis of rat brain or may merely prevent glycolysis from decreasing. Probably pyruvate is able to keep the tissue at its maximum glycolysis.

In muscle extract, glucose is broken down only when the glycolytic ferment of the muscle is aided by addition of hexokinase. The function of this activation is believed to be that of converting glucose into a special form available for esterification. The intermediary formation of phosphoric acid esters is an obligatory stage. Another type of glucose splitting may occur which is not accelerated by hexokinase, but only by a tumor extract which has been tentatively called coferment T.^{7*} No intermediary production of phosphoric acid ester appears to occur. Still a third activator, known as cozymase,[†] is present which is considered vital to the esterification reaction.

Since it had been demonstrated that gray substance could split glucose without phosphate being present, the activators which are involved in the formation of hexosephosphoric acid probably would not influence glycolysis. Actually, neither cozymase nor hexokinase show any activating effect, whereas coferment T induces production of from

* Coferment T is an enzyme extractable from tumor tissue which accelerates the splitting of glucose to lactic acid independent of the phosphorus concentration of the environing medium.

† Cozymase in some way assists the esterification of glucose. It has been shown to be adenyl pyrophosphate.

49-90 per cent more lactic acid than the control without ferment. As we shall see, however, this cannot be construed to mean that intermediary phosphoric acid esterification does not take place in brain.

Ashford and Holmes' experiments led them to believe that cerebral tissue has two mechanisms for production of lactic acid: The one, involving glycogen, requires the presence of phosphate, the other utilizes glucose and does not require the presence of inorganic phosphorus. The latter appears to be quantitatively the more important reaction. Presumably the limited ability of brain to produce lactic acid from glycogen is due to its relative inability to synthesize active hexose-phosphoric acid esters, which in turn may be due to the low concentration of coenzyme. Other investigators have published evidence which suggests that phosphoric acid esterification is a more important part of the glycolyzing mechanism of brain than had been supposed.

Metabolism of Lactic Acid.—Sodium lactate added to minced respiring brain substantially retards the decrease in respiration rate which normally occurs; whereas galactose, mannitol, sodium β -glycerophosphate and hexose di- and monophosphates are ineffective. This fact suggests the importance of lactic acid in the metabolism of nervous tissue. Sodium pyrophosphate added with lactate is more effective than lactate alone in the maintenance of respiration; while even better maintenance is secured by adding α -glycerophosphate.

The carbohydrate reserve of brain is very small; hence, during anaerobic survival, little extra lactic acid is produced unless glucose is added. The amount of lactic acid actually formed when no glucose is added appears to depend on the level of blood sugar at the time of death. During life, also, the brain seems to depend for its lactic acid precursor on sugar supplied by the blood stream, since the amount which can be found after death depends on the level of blood sugar at the time of killing. This fact is especially well illustrated when insulin is employed to alter the level of sugar in the blood. During hypoglycemia, brain lactic acid may be less than half its normal value; but if the animal is killed during the hyperglycemic state the values may be increased as much as 200 per cent. Lowering blood sugar by insulin has little effect upon the content of "reducing substance" in brain.

The brain not only normally absorbs hexose from the blood stream but also lactic acid. Impairment of oxidation in the brain by injection of sodium cyanide results in an increased production of lactic acid with outward diffusion into venous blood. The brain of diabetic as well as normal animals removes lactic acid from arterial blood. Since insulin is not required for its oxidation, the respiratory quotient remains at 1 even though insulin is no longer available.

Cerebral tissue of diabetic, like that of normal animals, is capable of converting glucose to lactic acid.⁸ Indeed, the brains of completely depancreatized cats exhibit resting lactic acid values closely corresponding to the degree of hyperglycemia at the time of death. It is

well that this is true, otherwise diabetic patients would doubtless fill our psychopathic wards.

Various anatomical portions of the central nervous system are found to contain widely varying amounts of lactic acid under anaerobic conditions, being as follows: Medulla > midbrain > cerebellum > cerebral hemispheres. The amount in other organs appears to be fairly constant, muscle ranging from 15–35 mg. per 100 Gm. of tissue, kidney 10–30 mg. and brain 50–70 mg. Compared with other tissues, brain is a large producer of lactic acid. Also, the rate of lactic acid formation in brain is greater than in other tissues.

Certain evidence indicates that oxygen regulates respiration of the body indirectly by determining the amount and kind of acid formed within the respiratory center, the principal acid possibly being lactic.

It must be pointed out that the recent discovery of "phosphagen," or creatine-phosphoric acid, has greatly modified the interpretation of the significance of lactic acid as a source of energy. The observation of Lundsgaard, that mono-iodoacetic acid could not only prevent postmortal lactic acid production but that it could also repress the formation of lactic acid in living muscle without interfering with its ability to contract, has shed new light on lactic acid metabolism.

Clinical Investigation of Carbohydrate Metabolism as Associated with Function of the Central Nervous System.—Much evidence derived from studies conducted on patients suffering from mental disease has shown that the carbohydrate metabolism is disordered. It will be recalled that the central nervous system is peculiar among most organs in being immediately dependent on sugar of the blood for its carbohydrate supply. Therefore, it does not seem unreasonable to assume that disorder of the systemic carbohydrate metabolism would be quickly reflected in the brain. The signs and symptoms following the administration of insulin lend strong support to this view.

Hyperinsulinism resulting either from excess administration or from a pancreatic neoplasm induces central nervous system symptoms of such protean character, that they are often confused with the more generally recognized psychoses. It is probable that substances which affect enzymes involved in degradation of sugar may similarly interfere with the carbohydrate metabolism of brain, and so produce manifestations which are not to be distinguished from those substances which are known to directly influence the level of sugar in the blood.

Determination of blood sugar in samples of blood drawn under uncontrolled conditions has served only to build up a large and exceedingly confusing literature. The glucose tolerance test is certainly more useful. McCowan and Quastel⁹ have introduced a convenient means of expressing the results of the tolerance test. They express the rise in blood sugar in terms of a hyperglycemic index:

$$\frac{(\text{Blood sugar two hours after glucose}) - (\text{the fasting level})}{(\text{Maximum blood sugar level}) - (\text{fasting level})} \times 100 = \text{Hyperglycemic index.}$$

The maximum blood sugar level is the maximum level to which the blood sugar rises within two hours after ingestion of glucose. The index represents the fraction of the peak rise, which still persists two hours after glucose is fed. Among manic-depressive patients, close parallelism is found between tolerance and emotional tension, the index being high when tension is high. Among schizophrenic patients, but few exhibit a high index. The benign stupor group usually have low indices. It is important to remember that old individuals normally show sustained hyperglycemia.

An interesting observation has been made which indicates that the hyperglycemic index is inversely proportional to blood cholesterol values and parallels the affective state, as determined by the psychogalvanometer. Apparently no amount of histrionic display on the part of a patient is capable of evoking a galvanic response unless there is emotional tension; and in such cases the hyperglycemic index is normal.

Still a third method for the study of the carbohydrate metabolism in man has been employed.¹⁰ Blood is simultaneously drawn from the carotid artery, jugular vein, brachial artery and basilic vein, and the amount of sugar determined in each sample, thus measuring removal or liberation of sugar by brain and muscle of the arm. More sugar is removed in the circulation from carotid artery to jugular vein than from brachial to basilic vessels. In dogs, at least, the arteriovenous difference is diminished but not eliminated by severe insulin hypoglycemia.

STEROLS IN THE BRAIN

That cholesterol is one of the principal constituents of brain tissue has been known since 1824, but its function in that organ remains as enigmatic as when discovered. Except for the adrenal cortex, no other organ contains such a large proportion of this substance.

Recent investigations have shown that cholesterol is only one member of a large group of vital substances. To the group belong such widely varying substances as vitamin D, carotene—the precursor of vitamin A—follicular hormone, a male sex hormone, bile acids, toad poisons and cardiac glucosides; all more or less related by a common structural heritage.*

Cholesterol is present to the extent of 0.5 per cent in five-month fetal brain, rapidly increases to 1.2 per cent in nurslings, and finally becomes stabilized at about 1.9 per cent in adults.¹¹ Ergosterol, the precursor of vitamin D, on the other hand, is at its maximum during fetal life and diminishes shortly after birth. This change is exactly the reverse to that in skin. During the fetal period ergosterol is present in but minute amounts, but after birth rapidly increases.

Dihydrocholesterol, a reduction product of cholesterol, is also found in brain in small amounts.¹² It is believed to be an end-product

* See Chapters IV and XXX.

in metabolism largely because it is secreted but not absorbed by the intestine. Coprosterol, a stereo-isomer of dihydrocholesterol, has not been found outside of the intestine and appears to be a product of bacterial reduction of cholesterol. Both vitamins D and A are found in small amounts in nervous tissue.

Metabolism of Sterols.—Cholesterol is absorbed to the extent of about 70 per cent of the amount ingested, and ergosterol to a considerably smaller extent.¹³ Phytosterols apparently contribute little to the body's store of sterol because of their poor absorbability. Absorption is aided by formation of complex "choleic acid compounds" with bile acids. Cholesterol exists free or esterified with fatty acids in the blood stream. It is probable that in some manner it aids in transport of fatty acids, since it tends to rise and fall with the amount of fatty acid present in the blood stream.

Normally, cholesterol is present in all cells of the body, and the amount contained is relatively fixed and characteristic for each tissue. Possibly because of its insolubility, it tends to fall out of solution and deposit as plaques in the vascular system or as gallstones. In general, when deposition takes place, the esters of cholesterol constitute a high percentage of the sterol deposit.

Excretion occurs through the medium of the bile; but much of this sterol is reabsorbed. That which is excreted by the intestine, and this constitutes the major portion, leaves the body in the stool. Most of the intestinal sterol becomes reduced to coprosterol.

The cholesterol balance may be positive or negative, depending on the amount already present in the body, the amount in the food and the ability to excrete sterol. Both synthesis and decomposition of cholesterol occurs but the reactions involved in either synthesis or degradation are unknown.¹⁴ Whether the brain acquires sterol from the blood stream or synthesizes its own sterol has not been investigated. Certainly the barrier between blood and spinal fluid shows little permeability for cholesterol.

Good evidence relates cholesterol chemically with bile acids. While in the test tube the close relationship may be demonstrated, in the animal body the link between the two has not as yet been established.

Esters of cholesterol are distributed in the body in a curious fashion. Bile, red blood cells and brain contain but traces of these compounds, while all other cells or fluids of the body contain an abundance. The significance of this fact remains unclear.

Irradiation of ergosterol produces vitamin D which plays a large part in regulating both calcium and phosphorus metabolism of tissues. Certain investigators believe that beside the vitamin itself other substances are produced as the result of irradiating, such as tachysterol and toxisterol, which may exert toxic effects in the body. Toxic effects manifest themselves among other signs as deposits of calcium in the media of blood vessels, in the kidneys, lungs, etc. The vessels

and cellular structure of the central nervous system seem peculiarly immune to the toxic action of these substances. In what manner a sterol could produce this striking action on calcium metabolism has received no satisfactory explanation.

Functional Significance of Cholesterol.—Certain suggestions regarding the function of cholesterol deserve mention, though it cannot be said that much support has been offered for these ideas.

Cholesterol may assist in maintenance of proper water balance of cells. It is believed to stand in antagonistic relationship with phosphatides; and the ratio of cholesterol to fatty acids is thought to determine the amount of water held by a cell. Some evidence suggests that sterols aid in oxidation of phosphatides *in vitro*. Possibly this has a bearing on the fact that the amount of cholesterol and phosphatide in muscle parallels the ability of muscle to accomplish work. Thus heart muscle, which must undergo the most rigorous activity, contains more phosphatide and sterol than voluntary muscle.

Cholesterol has both a protective function against hemolytic poisons and a detoxifying action for bacterial toxins. Whether these functions are exerted in the animal body remains purely speculative.

Associated Clinical Investigations.—Epileptic patients usually exhibit high phosphatide cholesterol ratios—during the period of seizure, which sink when the predisposition to attacks diminishes. The cholesterol level of blood does not change during periods when seizures are frequent, but the phosphatide rises.¹⁵

A relationship is believed to exist between the mental state and the blood cholesterol.¹⁶ Manic depressive and early dementia praecox patients exhibit increased values. By dividing the older case of dementia praecox into the emotionally heightened and those emotionally dulled it has been found that the former are characterized by decreased, and the latter by increased blood cholesterol. The total fatty acids exhibit similar trends. Other authors have questioned these findings.

Since it is known that adrenaline decreases plasma cholesterol, it has been suggested that emotionally heightened patients exhibit hyperadrenalinemia, and that the adrenaline is directly responsible for the hypocholesterolemia. The amount of cholesterol in the blood may bear an inverse relationship to the hyperglycemic index.

FATTY ACIDS AND PHOSPHATIDE OF BRAIN*

Fatty acids in the brain are present in an overwhelming excess combined as phosphatide and in much smaller quantities as cerebroside, sphingomyelin and neutral fat. Except for the presence of cerebronic acid and acids of the C₂₂ series, brain contains the fatty acids ordinarily found in other tissues. The average molecular weight of fatty acids from brain is considerably higher than acids from other organs.¹⁷

Fatty acids probably contribute to covering the energy require-

* Refer also to Chapters III and XXI.

ments of the brain. Proof for this statement is not at hand; but in view of the fact that the mechanism of the destruction of lipid is unknown, we must not be too ready to believe that the more easily demonstrable carbohydrate catabolism can account for the total energy balance.

The phosphatides or phospholipids of brain are largely made up of cephalin and lecithin with smaller amounts of sphingomyelin. It will be recalled that the base combined in lecithin is choline, and that in cephalin it is amino-ethanol, while in sphingomyelin both choline and sphingosine are found. There seems little doubt that the names "lecithin" and "cephalin" connote groups of similar substances differing possibly in stereochemical configuration or in the fatty acids combined with the glycerol of the molecule. Brain phosphatides contain all of the even-numbered fatty acids from C_{16} to C_{24} . Of the saturated acids, palmitic and especially stearic predominate. Among the unsaturated acids, C_{22} acids are especially prominent compared with the amount of these acids found in other organs.^{18, 19}

Phosphatides are unique among lipids in being soluble both in water and in organic solvents. Their composition is also unusual, containing as they do fatty acids both saturated and unsaturated, glycerol, phosphoric acid, amino-ethanol and the pharmacologically active base choline. Enzymes attack the molecule with relative ease. Thus, an enzyme in cobra venom splits off one molecule of fatty acid yielding so-called "lysophosphatide." This substance is a powerful hemolytic poison. Phosphoric acid is removed from the molecule by one of the phosphatases and lipase is able to free the fatty acids.

Metabolism of Phosphatide.—It is improbable that phosphatides are absorbed without first being split by the digestive juices. Certain evidence indicates that they are resynthesized by the intestinal mucosa and poured into the lymph and blood vessels. Water solutions of phosphatide may be injected parenterally. A few hours after such an injection, phosphatide in the blood returns to normal and that in the liver is markedly increased. Cerebral tissue, unlike other tissues, does not increase its content of phosphatide.²⁰ Indeed, this organ is unusual in that the quality of phosphatide fatty acids is independent of the diet.²¹

Except in brain and liver, there are no large deposits of phosphatide. Apparently, the body converts them into neutral fat if storage is desirable. No conversion of phosphatide into sugar has ever been convincingly demonstrated.

The phosphatide molecule does not absorb oxygen more readily than the free fatty acids contained therein; indicating that, at least in so far as oxidation is concerned, esterification with glycerophosphoric acid does not increase its oxidizability. Iron salts powerfully catalyzes oxidation, and to a considerably greater extent than other heavy metals. Both hydroxy- and keto-acids result from such oxidation.

While enzymes are present within the body which have the power of cleaving the phosphatide molecule, no such reaction has been identified. Among these enzymes may be mentioned, phosphatase (which splits off phosphoric acid), lipase (which splits off fatty acids), and cobra venom (which splits off the unsaturated fatty acid). Products of great toxicity (such as lysolecithin) may result from such enzymatic action.

Lipid Equilibria.—It is not possible within the limits of this chapter to discuss the evidence for the existence of equilibria among the lipids. Suffice it to state that they probably exist and furnish the best means of describing so-called "primary lipid disorders."²² Among these are grouped the Hand (Schüller-Christian) syndrome, Niemann-Pick's disease, Gaucher's disease, xanthomatosis and Tay-Sachs' amaurotic familial idiocy. These diseases are of special interest in regard to the biochemistry of brain because most of them have cerebral components. Furthermore, since each disease is characterized by the irreversible deposition of one of the lipids, a means is at hand which will further knowledge of intermediary metabolism of these enigmatic substances.

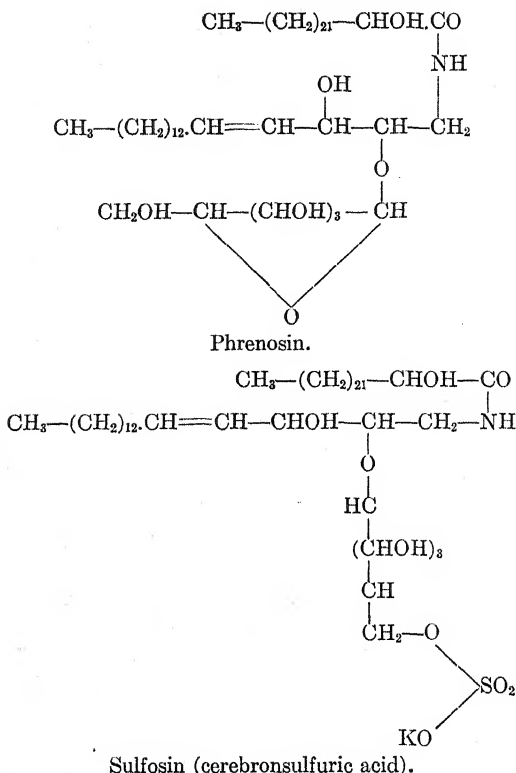
We know but little of the agents (such as hormones and enzymes) which regulate lipid equilibria. It has become increasingly evident that there are definite regulatory substances which affect fat metabolism as surely as insulin and adrenaline regulate sugar metabolism. Indeed, the substances which act on carbohydrate metabolism without exception directly affect lipid metabolism. Insulin lowers the blood fat and cholesterol just as convincingly as it does sugar. Thyroxine and adrenaline both reduce the level of blood cholesterol; and yet we have not accustomed ourselves to think of these hormones as fat-metabolizing hormones.

Discussion of the oxidation and intermediary metabolism of the fatty acids will receive no discussion here (see Chapter XXI). It will be recognized that the "lactic acid of carbohydrate metabolism" has not as yet found its counterpart in lipid metabolism.

Cerebrosides.—Cerebrosides are substances of glucoside nature which, on hydrolysis, yield a reducing sugar (galactose), a base (sphingosine) and a fatty acid.²³ Five cerebrosides have been adequately characterized, though certain of them may prove to be mixtures of closely related substances; these are, phrenosin, kersasin, nervon, oxynervon and sulfosin (cerebronsulfuric acid).

While cerebrosides are found in other organs, especially liver, spleen and heart, the amount as compared with that in brain is exceedingly small. Nothing is known of the metabolism of this group of substances. In view of their interesting structure, many possibilities suggest themselves as to the rôle they might play in either carbohydrate or lipid metabolism.

There is one anomaly of metabolism at least which involves cerebroside, namely, Gaucher's disease. Cerebroside gorges the cells of



the spleen and liver and causes enormous enlargement of these organs. Too little is understood of the origin and fate of cerebrosides to attempt any explanation of the etiology of this disease at present.

NITROGENOUS METABOLISM

Few investigators have concerned themselves with the nitrogen metabolism of the brain. Scattered studies have been made of nitrogen in the urine and blood of patients suffering from mental disease, but aside from these the field has remained almost barren.

Creatine and creatinine are both found in brain tissue. Especially is the creatine content of cerebellum high as compared with the hemispheres.²⁴ Gray matter is far richer in this substance than white. Fasting and creatine feeding do not alter the creatine content of brain. It is considerably increased in the blood of catatonic patients showing marked rigidity; whereas low values are found in cases of deteriorating dementia praecox and involutional melancholia.

The nonprotein nitrogen of rat brain is surprisingly constant, especially if the rats are kept under uniform nutritional conditions. After feeding, a rise occurs which reaches a maximum in two to three hours. Severe exercise also causes a marked increase.

The ratio of nonprotein nitrogen to total nitrogen has been employed as a method for differentiating various anatomical portions of the brain. The results appear promising but cannot as yet be accepted without further investigation.

The urea content of brain is apparently more subject to fluctuation than nonprotein nitrogen, if the published data are correct. The amount in the brain closely simulates that of blood. This is strikingly illustrated in cases of uremia, where the urea may rise to a high level in the brain but never to the extent to which it rises in blood.

Ammonia is also present in nervous tissue, presumably originating from adenylic acid. Slight trauma incurred by removing samples of brain for analysis greatly increases the ammonia content. Insulin lowers and anoxemia increases the ammonia. Glucose tends to depress its formation both under aerobic and anaerobic conditions.

Proteins of Brain.—A substance similar to, but not identical with true keratin has been isolated from brain. This so-called "neurokeratin" contains histidine, lysine and arginine.²⁵ The nucleic acid portion of the nucleoprotein does not appear to differ essentially from that of other tissues. The nucleoproteins are species-specific but not organ-specific.

An organ-specific haptene appears to have been separated and partially purified from brain. It is demonstrable in nervous tissue from all higher animals by use of the antigen-antibody reaction.²⁶

Most of the ordinary amino acids have been found to be constituents of the proteins from brain; but in different anatomical portions distribution of amino acids varies considerably.

The glutathione content of blood does not appear altered in cases of melancholia. Brain contains this substance in amounts similar to that found in kidney and heart but far less than in liver.

GASES AND ELECTROLYTES

Intracellular Hydrogen Ion Concentration.—The only studies of intracellular hydrogen ion concentration of the central nervous system of which we are aware are those of Chambers and Pollock on the giant nerve cells from the medulla of goose fish.²⁷ The cytoplasm of these cells exhibited colorimetric pH value of 6.9 whereas the nuclear pH was not below 7.6. After so short a sojourn as ten to fifteen minutes in normal fish serum the nerve cells appeared as normal as before; nevertheless the pH difference between nucleus and cytoplasm had become neutralized.

Observation of the pH of blood and spinal fluid in mental disease indicates no variation from the normal.

Respiratory Stimulation of Carbon Dioxide and Mental Disease.—Loevenhart, Lorenz and Waters have made the interesting observation that rebreathing of 30 per cent carbon dioxide-air mixtures leads to a short period of nearly normal mental function in cases of catatonic

schizophrenia.²⁸ The mute and inaccessible patients respond in the most satisfactory manner. The authors regard the phenomenon as a true stimulation of parts of the cortex much as the medulla may be stimulated. Mere oxygen want cannot be responsible for the reaction, because essentially the same effect can be elicited by injection of sodium cyanide. The difficulty appears to be that cells of the psychotics' brain cannot utilize the oxygen which is present in abundance.

Several physiologic changes are induced by carbon dioxide inhalation which might be held responsible for the mental excitation; namely, acidosis, high CO_2 content, loss of consciousness, increase in ventilation, nonspecific shock and increased cerebral circulation. It has not been possible to decide which of these are the most important factors.²⁹

It has been affirmed that while normal individuals respond to inhalation of CO_2 by increased respiratory ventilation, psychotics, especially those in catatonic stupor, usually do not. This, and other data, has led to the conclusion that the excitability of the respiratory center is depressed.³⁰ This depression of the center does not appear merely as a symptom of the catatonic stupor, since some of the patients were restless and excited, while others might have passed for normal in their conduct without a careful psychiatric examination.

Chloride and Bromide.—The normal content of chloride varies in different portions of the brain. Gray matter is richer than white, and medulla, thalamus and pons are relatively low in chloride. In mental disease there is often marked chloride retention, but it is difficult to determine whether this observation has any intimate association with the metabolism of brain or is only a secondary effect incident to the poor physical condition of the patient. In patients suffering from uremia, the chloride content may be increased four to seven times in gray and two to three times normal in white matter. Chloride hunger reduces the chloride content to a considerable extent.

Due to imperfect methods the bromide content of brain is not accurately known. Recent claims have been made that in cases of manic depression the content of bromine in the blood is markedly diminished. The lowering in bromine is believed by some investigators to be especially pronounced in patients suffering from psychoses in which psychomotor unrest is marked. As overactivity disappears, the blood bromine returned to normal. The accuracy of these results has been questioned.

Sodium bromide fed by mouth is absorbed largely by muscle and skin, very small amounts appearing in the brain. However, cortex absorbs almost twice as much as basal ganglia, cerebellum, or white matter. The distribution is quite different from that of sodium chloride.

Iodine.—The midbrain is rich in iodine while the hemispheres are poor in this element.³¹ Thyroxine, orally or intravenously administered, increases the iodine content of the midbrain especially, whereas potassium iodide or diiodotyrosine is ineffective. Removal

of the thyroid gland causes a rapid fall in the iodine content of the brain; in the midbrain the lowest level is reached within forty-eight hours, only a trace remaining in some cases. Administration of thyroxine rapidly restores the original level. It is believed by Schittenhelm and Eisler that the source of the blood iodine is the thyroid gland and the musculature, while both the production and utilization are under control of the central nervous system.³¹

Calcium.—The calcium of brain is mobile to a great degree, a relatively large elevation and lowering occurring without recognizable clinical effects. The physical state of the calcium is not known, nor have the changes been correlated with variations in other electrolytes, more especially phosphorus, except in rare instances. It is, therefore, impossible in our present state of ignorance to give any satisfactory interpretation of the scattered results which have been published.

The content of calcium in the blood clearly affects the metabolism of the central nervous system. Excessive amounts depress the irritability of the brain while reduction below normal probably increases irritability. It is not meant to imply that this is or is not a direct effect of calcium, as evidence from many sources indicates that simultaneous alteration in acid-base and phosphorus equilibria may play an equally important part. Acute calcium poisoning appears to result in enrichment of circumscribed areas in the brain, especially midbrain and cerebellum.

The calcium and phosphorus contents of most organs of the bodies of rachitic rats are normal. The brain of normal rats contains approximately 182 mg. per cent of calcium, and 1278 mg. of phosphorus; while brain of rachitic animals average only 59 mg. calcium but about normal total phosphorus.³² During this disease the calcium content of blood and that of brain are altered independently of one another. Removal of the parathyroid glands reduces the calcium of blood but not that of brain; hence it is the rickets rather than the tetany which leads to withdrawal of calcium from the brain. Marked reduction of calcium in the diet may also reduce the cerebral calcium, phosphorus remaining normal. Antirachitic treatment brings about evidence of bone healing long before the calcium content of the brain is restored to normal.

Intravenous injection of large amounts of viosterol in dogs leads to calcium increase of over 100 per cent in brain. The phosphorus is affected in a much less constant manner, if at all.

Many investigations have been concerned with the relationship of calcium to narcosis. The localized injection of calcium into the region of the infundibulum induces sleep, while potassium salts cause excitement. The calcium content of blood, on the other hand, is lowered during narcosis: The longer the period of sleep the greater the depression of calcium. The autonomic nervous system exerts a regulatory action on blood electrolytes; and it may well be that changes in the blood calcium are the result of impulses arising in the hypothalamus.

Curiously, neither the calcium nor magnesium content of the brain is appreciably altered during magnesium sulfate narcosis.

The blood from patients in manic states usually exhibits increased amounts of calcium and phosphorus, while depression tends to decrease these electrolytes.

Arsenic.—Apparently, surprisingly little arsenic is stored in brain after arsphenamine treatment, although individual variation in this regard is large. Very minute amounts may be found occurring normally.

Tellurium and Selenium.—These metals probably do not occur as normal constituents of brain. Colloidal metallic selenium and tellurium injected intramuscularly appear after a short time as a heavy deposit of metal in the gray substance of the brain, the white matter remaining entirely free.³³ Brain tissue *in vitro* is able to reduce selenious or tellurous acids with deposition of the metal in the gray matter. This power is lost if the tissue is previously heated to 70°. Presumably the reaction is enzymatic.

Lead.—While lead is usually stored to the greatest extent in bone, occasionally the brain participates. Organic lead compounds, such as tetra-ethyl lead, employed as antiknock in gasoline, is especially prone to accumulate in the central nervous system. Aub holds the view that the lesion in lead palsy is in the muscle and that the nerve is only secondarily involved.³⁴ Involvement of the meninges may be more important than that of the brain in meningo-encephalopathy.

Mercury.—Even after administration of soluble mercury salts, the brain contains but minute amounts of mercury.

Alumina, Silica and Iron.—Adult human brain contains about 0.77–0.45 mg. per cent of aluminum and human fetal brain 22 mg. per cent of silica.³⁵ Iron is increased in cases of hemochromatosis and general paresis. It is stated to be altered in mental disease.

Manganese and Magnesium.—These metals are both present in brain. They are especially interesting when not present. Manganese deprivation leads to loss of maternal instinct in rats. Hyperirritability, trophic disturbances and fatal convulsions characterize the symptoms and signs of magnesium deprivation. The magnesium content of the fetal brain is greater than that of any other fetal organ.³⁶

Other Metals.—Copper is present to the extent of 4–6 mg./1000 Gm. of adult brain and zinc 5–15 mg. Exceptionally large amounts of zinc appear to occur in fetal brains. In patients suffering from Wilson's disease, the copper content of both the liver and the basal ganglia has been found raised. Bismuth, rubidium and tin have all been observed to occur in normal brain tissue.³⁷

PHYSICAL CHEMISTRY

Mention may be made of certain problems associated with the metabolism of the brain which are largely physicochemical in nature. As an example, miners working in a hot, humid atmosphere perspire freely,

with the result that they lose salt and water in large amounts. If they allay their thirst with tap water, they are subject to violent cramps and temporary mental derangement. All these signs and symptoms may be avoided by drinking water containing sodium chloride. Whatever the explanation of this phenomenon may be, it suggests that relatively simple upset in salt-water equilibria of the blood and tissues is responsible for profound mental changes.

Changes in Water Metabolism as Indicated by Brain Swelling.—

The brain is enclosed in a rigid bony case with only about 10–16 per cent of free fluid space. Hence there is relatively little room for change in volume of either brain, cerebrospinal fluid or blood. Swelling of the brain may occur either from absorption of fluid which remains extracellular—the so-called “wet brain”—or the fluid may be intracellular, producing a dry swollen organ. It remains uncertain whether water in the “dry brain” is really intracellular or whether it is not in the bound state; that is to say, in such association with colloids and crystalloids that its dissolving power is zero.³⁸

Water may be abstracted from brain by intravenous injection of hypertonic electrolyte solutions, or it may be added by injection of hypotonic solutions. This fact has been made the basis of therapeutic procedures of great importance.³⁹

Different ions each have a more or less characteristic inhibitory effect on the swelling not only of cerebral tissue as a whole but on its separate anatomical portions. This problem is one of great complexity and importance, but as yet has received scant attention.

Heat applied to the body in the form of hot baths causes the blood to become more dilute and the brain volume to decrease. Certain evidence suggests that heat generated internally from metabolic sources influences brain volume in a different manner than externally applied heat; for in fever, particularly in children, the brain volume is believed to be increased.

Water Intoxication.—Signs and symptoms of severe intoxication result when excessively large amounts of water are ingested.⁴⁰ Intoxication may be acute, ending in death in from four to twelve hours; or subacute, lasting several days. Increased intracranial pressure is observed which is believed to be due to disturbance in salt-water equilibria of the brain with edema formation. The syndrome may be prevented or alleviated by the timely administration of hypertonic saline. Cases of diabetes insipidus may exhibit symptoms not unlike those seen in water intoxication.

Desiccation of the body, at least in the frog, results in loss of water from the skin, spleen, liver and blood; while the eyeballs, lungs and brain lose but little of their water.

Electrolyte Antagonism.—The phenomenon of electrolyte antagonism is especially well illustrated by the effects of magnesium chloride and calcium chloride on the central nervous system. Narcosis results from injection of the magnesium salt; and the narcosis may

be almost instantaneously abolished by injection of calcium salts. Potassium is also said to be an effective antidote.

It is possible that magnesium shifts the balanced emulsions—which is believed, at least by certain investigators, to constitute the kinetic surface membrane—to one in which “oil” is the outside and water the internal phase. The permeability may thus be lowered for water-soluble gases and other metabolites, resulting in narcosis. The simplicity of this explanation has much that is attractive, but it does not contain the whole truth.

Water Balance in Morphine Addiction and Epilepsy.—Well advanced morphine addiction in animals leads to dehydration of the brain, liver and kidneys. Sudden withdrawal of the morphine induces a remarkable redistribution of water in the body. The blood, spleen and surface tissues all lose water, while edema formation occurs in brain, muscle, liver and kidney. It is possible that the edema of the brain may be associated with the withdrawal symptoms which are so alarming.

Sudden increase in water intake or administration of pituitrin may precipitate convulsions in previously dehydrated epileptic patients. Sufficient urea added to the water, however, may prevent occurrence of the seizure. The effect of water does not seem to be due to production of cerebral edema, since the spinal fluid pressure remains normal. Ordinary intercellular brain edema is not regarded of great importance in causation of epileptic seizures.⁴¹ Entrance of water, however, into the cells in excessive quantities may be extremely significant; indeed, certain data indicate that the barrier normally preventing free passage of water and various solutes into and out of brain cells function inefficiently in epileptics.

DIET AND DEGENERATION OF THE NERVOUS SYSTEM

Deficiencies in diet may be reflected in the central nervous system by the appearance of progressive degenerative changes. These changes result in both fetal or adult organisms and may vary according to the stage to which the nervous system has developed. It is not difficult to believe that many lesions of the brain have their origin in inadequate nutrition—lesions which might easily be classified as constitutional.

Vitamin B Complex Deficiencies.—Dogs fed on a diet deficient in both antineuritic B₁ and pellagra preventing B₂ vitamins develop loss of appetite, vomiting and spastic paralysis.⁴² The syndrome progresses rapidly to convulsions, coma and death. All animals deprived of the complex suffer from diffuse, irregular loss of myelin, chiefly in the cord and less marked in the cerebral cortex. Deficiency of the thermostable B₂ factor causes the appearance of large zones of degeneration in the vermis and the region of the substantia gelatinosa Rolandi.⁴³ Lesions have been produced in adult dogs similar to those observed in patients suffering from pellagra by feeding diets deficient in vitamin B₂.

A number of investigators believe that the tissues of vitamin B₁ deficient animals lack the ability to oxidize foodstuffs properly. It has been pointed out that, at least in the pigeon, increased production of lactic acid occurs, especially in the lower portion of the brain at, or approaching the period of opisthotonic convulsions. The mechanism for oxidation of lactic, however, appears to remain efficient.

Brain tissue from polyneuritic animals exhibit *in vitro* lowered power of oxygen uptake in presence of glucose.⁴⁴ This was found to be true, when symptoms were prolonged, in all parts of the brain except cerebellum. The respiratory quotient which ordinarily is close to unity, is reduced in B₁ deficient pigeons and dogs.

Addition of small amounts of antineuritic yeast concentrates to brain tissue of polyneuritic pigeons suspended in glucose-phosphate solution effects partial restoration of the lowered power of oxygen uptake. While increasing the oxygen uptake (especially in the presence of added lactate), addition of vitamin causes no significant increase in the amount of lactate removed. Such tissue normally readily destroys lactate and calculation shows that the amount destroyed is sufficient to require at least the total oxygen absorbed by the tissue for its complete oxidation. B₁ concentrate fails to restore oxygen uptake when sodium pyruvate is employed as substrate. This has been construed to mean that pyruvic acid is not an intermediary stage in the lactic acid oxidation of pigeon brain.

It is interesting to note that oxygen uptake of cerebellum is especially involved; since many of the signs and symptoms probably originate in the nuclei of this region of the central nervous system.

Lesions in the nervous system have been described in cases of pellagra. The water content of the brain is increased while protein and lipid are decreased. Associated with the extensive tissue degeneration, cerebroside, phosphatide and "sulfatide" are also reduced.

Vitamin A Deficiency.—Diets containing a large amount of cereal and deficient in vitamin A or carotene cause, in puppies, degenerative changes in the spinal cord in the form of demyelination of nerve fibers.⁴⁵ Addition to the diet of ergot hastens and intensifies these changes. The addition of carotene or vitamin A prevents or diminishes these degenerative changes even when ergot is eaten. Spinal cord degeneration of this type does not seem to develop until the reserves of vitamin A in the liver are dispersed. Since these stores may be very large in well-fed animals, the time of onset of symptoms of spasticity, incoordination and weakness varies greatly in different animals. Signs, symptoms and locus of degenerative changes following vitamin A deprivation appears very different in different animal species.

Certain evidence indicates that the placenta is not easily permeable to vitamins A and D or its precursors. It seems possible that the fetal organism must synthesize its own vitamin, but this important question has not as yet been satisfactorily answered.

Vitamins C, D and E.—Deficiency of vitamin C produces a condition characterized by extreme nervous instability. As yet, no chem-

ical studies appear to have been made in this condition. Decrease in the calcium of the brain has been observed in vitamin D rachitic animals, but no characteristic histologic lesions have been observed in this deficiency.

Remarkably sudden onset of what is probably upper motor neurone paralysis occurs in suckling young when the lactating mother is deprived of the antisterility vitamin E.⁴⁶

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REFERENCES

1. Holmes, E. G., and Holmes, B. E.: *Biochem. J.*, **19**, 492 and 836 (1925).
2. Toennissen, E., and Fischer, R.: *Z. physiol. Chem.*, **161**, 254 (1926).
3. Embden, G., Deuticke, H. H., and Kraft, G.: *Klin. Wochschr.*, **12**, 213 (1933).
4. Meyerhof, O.: *Nature*, **132**, 337 (1933).
5. Warburg, O., Posener, K., and Negelein, E.: *Biochem. Z.*, **152**, 309 (1924).
6. Dickens, F., and Greville, G. D.: *Biochem. J.*, **27**, 1134 (1933).
7. Bumm, E., and Fehrenback, K.: *Z. physiol. Chem.*, **195**, 101 (1931).
8. Himwich, H. E., and Nahum, L. H.: *Am. J. Physiol.*, **88**, 680 (1929); **90**, 389 (1929).
9. McCowan, P. K., and Quastel, J. H.: *J. Ment. Sci.*, **77**, 525 (1931).
10. Myerson, A., and Halloran, R. D.: *Am. J. Psychiat.*, **10**, 389 (1930).
11. Page, I. H., and Menschick, W.: *Biochem. Z.*, **231**, 448 (1931).
12. Schönheimer, R., and Hrdina, L.: *Z. physiol. Chem.*, **212**, 161 (1932).
13. Page, I. H., and Menschick, W.: *Biochem. Z.*, **221**, 6 (1930).
14. Page, I. H., and Menschick, W.: *J. Biol. Chem.*, **97**, 359 (1932).
15. McQuarrie, I., Bloor, W. R., Husted, C., Patterson, H. A.: *J. Clin. Investigation*, **12**, 247 and 255 (1933).
16. Stenberg, S.: *Acta Med. Scand.*, **71**, 1 (1929).
17. Brown, J. B.: *J. Biol. Chem.*, **83**, 783 (1929).
18. Klenk, E., and Schoenebeck, O. V.: *Z. physiol. Chem.*, **194**, 191 (1931).
19. Page, I. H., and Rudy, H.: *Z. physiol. Chem.*, **205**, 115 (1932).
20. Pasternak, L., and Page, I. H.: *Biochem. Z.*, **252**, 254 (1932).
21. Sinclair, R. G.: *J. Biol. Chem.*, **86**, 579 (1930); **95**, 393 (1932).
22. Sobotka, H.: *Naturwissenschaften*, **18**, 619 (1930).
23. Klenk, E.: *Die Chemie der Cerebroside und Phosphatide* (1930).
24. Harding, V. J., and Eagles, B. A.: *J. Biol. Chem.*, **60**, 301 (1924).
25. Block, R. J., and Vickery, H. B.: *J. Biol. Chem.*, **93**, 113 (1931).
26. Rudy, H.: *Klin. Wochschr.*, **12**, 433, 1100 (1933).
27. Chambers, R., and Pollock, H.: *J. Gen. Physiol.*, **10**, 739 (1927).
28. Loevenhart, A. L., Lorenz, W. E., and Waters, R. M.: *J. Am. Med. Soc.*, **92**, 880 (1929).
29. d'Elseau, F. C., and Solomon, H. C.: *Arch. Neurol. Psych.*, **29**, 213 (1933).
30. Golla, F. L.: *Proc. Roy. Soc. Med.*, **22**, 31 (1929).
31. Schittenhelm, H., and Eisler, B.: *Klin. Wochschr.*, **11**, 9 (1932).
32. Hess, A. F., Gross, J., Weinstock, M., and Berliner, F. S.: *J. Biol. Chem.*, **98**, 625 (1932).
33. Jahnel, F., Page, I. H., and Müller, E.: *Z. ges. Neurol. Psych.*, **142**, 214 (1932).
34. Aub, J. C., Fairhall, L. T., Minot, A. S., and Reznikoff, P.: *Medicine*, **4**, 1 (1925).
35. King, E. J., Stantial, H., and Dolan, M.: *Biochem. J.*, **27**, 1002 (1933).
36. McCollum, E. V., and Örent, E. R.: *Science*, **73**, 507 (1931).
37. Sheldon, J. H., and Ramage, H.: *Biochem. J.*, **25**, 1608 (1931).
38. Gortner, R. A.: *Trans. Faraday Soc.*, **16**, 678 and 686 (1930).
39. Weed, L. H., and McKibben, P. A.: *Am. J. Physiol.*, **48**, 512 and 531 (1919).
40. Rowntree, L. G.: *J. Pharmacol.*, **29**, 135 (1926).
41. McQuarrie, I., and Peeler, D. B.: *J. Clin. Investigation*, **10**, 915 (1931).
42. Gildea, E. F., Kattwinkel, E. E., and Castle, W. B.: *New Eng. J. Med.*, **202**, 523 (1930).
43. Zimmerman, H. M., and Burack, E.: *Arch. Path.*, **13**, 207 (1932).
44. Gavrilesco, N., and Peters, R. A.: *Biochem. J.*, **25**, 1397 (1931).
45. Mellanby, E.: *Brain*, **54**, 247 (1931).
46. Evans, H. M., and Burr, G. O.: *J. Biol. Chem.*, **76**, 263, 273 (1928).

CHAPTER XXX

HORMONES

HORMONES are substances produced by "ductless glands." These hormones are discharged into the blood and stimulate some organ or organs to activity. The glands without ducts ("ductless glands") are often called "endocrine glands"—glands, that is, having an "internal secretion." The word "hormone" itself comes from a Greek work meaning "to excite."

The first clear statement dealing with the ideas involved in an "internal secretion" we owe to that great French physiologist, Claude Bernard, who published his results in 1850 in connection with his studies of the conversion of liver glycogen into glucose. At about the same time, the English physician, Thomas Addison, described in masterly fashion a disease of the adrenals which is still known as "Addison's disease." In the eighties of the last century, T. Kocher described myxedema as due to the loss of the functional activities of the thyroid; M. Schiff performed epoch-making experiments on the removal of the thyroid; P. Marie discovered that acromegaly is a disease due to the pituitary; and Brown-Séquard, with somewhat questionable means, founded the science (shall we call it such?) of organotherapy—treatment by means of glandular extracts. A notable discovery of this era we owe to J. von Mehring and O. Minkowski, who showed that the complete removal of the pancreas results in severe diabetes. In the nineties of the last century T. Gley connected tetany with the removal of the parathyroids; E. A. Schafer showed how an injection of an extract from the adrenals increased blood pressure; and E. Baumann discovered that iodine is a normal constituent of the body. In 1902 W. M. Bayliss and E. H. Starling isolated a "secretin" involved in the mechanism of pancreatic secretion, and the very word "hormone" came into being.

More recent still, in point of discovery, is the exhaustive work on adrenaline, insulin, thyroxine, the cortical hormone, and the sex hormones; these will be taken up in the various sections of this chapter.

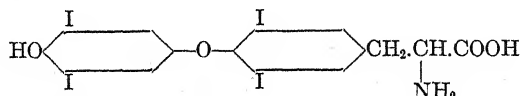
THE THYROID HORMONE

The thyroid is the great regulator of the *rate* of metabolism in the body. Its hyperactivities may be associated with such a disease as exophthalmic goiter and its hypodeficiencies may be connected with cretinism in the young and myxedema in the more mature.

Iodothyroglobulin.—This substance with many of the properties of a globulin was first isolated by Oswald in 1899, and probably has the physiologic activity of the entire gland. It is regarded by many

today as the real thyroid hormone. It may be obtained by extracting the gland with physiologic salt solution and precipitating the hormone by half saturating the extract with ammonium sulfate. Several modifications in its preparation have been suggested.¹

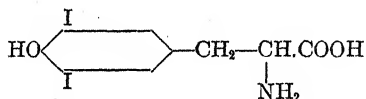
Thyroxine.—Using a method of alkaline hydrolysis, Kendall was the first to succeed in isolating a protein-free substance having, presumably, most if not all of the properties of the entire gland.² To it he gave the name *thyroxine*. The correct chemical formula for thyroxine, as well as a method of synthesizing it, we owe to Harington.^{3, 4} It may be regarded as a tyrosine derivative:



being β -(3: 5-diiodo-4(3': 5'-diiodo-4'-hydroxyphenoxy) phenyl- α -aminopropionic acid. (The synthesis of thyroxine is given on p. 168.)

The racemic compound obtained on hydrolysis of the gland can be resolved into the *d*- and *l*-forms, and it can be shown that the *l*-modification is three times more active than the *d*-.

3,5-Diiodotyrosine.—This substance with the formula



has also been isolated from the thyroid gland.^{3, 5} While it accelerates metamorphosis in amphibia, it has no effect on the basal metabolic rate—so unlike thyroxine. Though adequate proof is lacking, the substance is considered as a probable precursor of thyroxine itself.*

Physiologic Tests for Thyroid Activity.—The metamorphosis of tadpoles is hastened by feeding them thyroid gland (or thyroxine, etc.). The shortening of the tail during metamorphosis has been used for quantitative studies of thyroid activity. The effect on basal metabolism is perhaps even more important for test purposes. The injection of 1 mg. of thyroxine to a patient suffering from myxedema will increase the basal metabolism by 2.8 per cent above the normal value. Still another test has been devised by Reid Hunt, who has shown that thyroxine increases the resistance of mice to the action of acetonitrile.^{4, 6}

Thyroidectomy.—After removal of the thyroid in young animals, there is retarded growth and delayed ossification. "The generative organs remain relatively small and the ova and spermatozoa may not come to maturity." The basal metabolism is markedly lowered and "there is a marked lack of intelligence."⁶

Feeding Thyroid.—Animals suffering from a lack of thyroid and cretinous children suffering from hypothyroidism are markedly helped

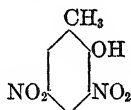
* See p. 168.

by the feeding of thyroid extract and by the feeding or, better still, the injection of thyroxine. There is marked physical, if somewhat less mental, improvement, and the basal metabolic rate usually tends to return toward normal.

Basal Metabolic Rate.—It is common for hypo- and hyperthyroid cases to show values of less than 20 per cent below normal (-20) or 20 per cent above normal ($+20$), respectively. A valuable diagnostic instrument is the change in metabolic rate in the direction of "normalcy" as improvement in the patient is registered.

Thyroid Diseases.—At present there is much confusion with regard to classification. Graves' disease, with its accompanying exophthalmos, is usually taken as typical of the hyperthyroid condition. The cretin and the myxedematous patient typify, to some extent, the hypothyroid condition. Included in the latter, perhaps, is the patient with endemic goiter, "which is an enlargement of the thyroid gland but not associated with toxic features," and where the addition of iodine (in the form of one of its salts) to the diet has beneficial results.*

The Nitrophenols and Basal Metabolism.—The dinitrophenols and, more particularly, dinitro-*o*-cresol,



have powerful effects in increasing the metabolic rate. Dodds has shown that while the injection of dinitrocresol into a myxedematous patient raises the basal metabolism considerably, his myxedema remained unaffected; thereby proving that thyroxine acts "in a manner quite apart from its mere stimulation of metabolism."⁷

THE PARATHYROID HORMONE

Attached to the thyroid in the neck are four smaller organs called *parathyroids* ("para" meaning "near"). For some time these glands were not sharply distinguished from the thyroids themselves. The French physiologist, Gley, had much to do in connecting the extirpation of these glands with tetanous convulsions; and MacCallum and others showed how these tetanic convulsions were accompanied by a fall of blood calcium. An active parathyroid extract was prepared, among others, by Collip, who has published the most extensive studies in this field.

Parathyroid Extract.—Collip has prepared the extract by acid hydrolysis of the parathyroids of the ox. The injection of this extract prevented tetany in parathyroidectomized dogs. Furthermore, the blood calcium level, low in the beginning, was restored to normal. He also made the observation that an overdose of the extract gave rise

* The production of goiter in rabbits by the feeding of cabbage has been accomplished by Marine and others. The basal metabolism of such rabbits was 18-20 per cent below that of normal controls.⁶

to a marked hypercalcemia; in many cases from a normal of 10.5 mg. per 100 cc. to as high as 20 mg.^{8, 4}

The increase in blood calcium upon the injection of the hormone extract is now the basis of quantitative evaluation. Collip has defined his unit as one one-hundredth of the amount required to produce a 5-mg. rise in blood serum calcium in fifteen hours in a dog of 20 Kg.

This hormone—sometimes called *parathormone*—has not yet been isolated in the chemically pure state. In its impure condition it shows certain protein characteristics. Its relation to vitamin D in so far as calcium metabolism is concerned is not very clear.⁵⁵

INSULIN

Insulin is the active principle present in the pancreatic gland which plays a dominant rôle in carbohydrate metabolism. In 1885 Mehring and Minkowski showed that the removal of the pancreas of dogs was followed by a severe diabetes. Since that time many have been the attempts to prepare an active extract. This was finally accomplished by Banting and his associates in 1921.

The Banting Group.—In the beginning an acid alcoholic extract of the pancreas was made. These extracts very definitely reduced the blood sugar in diabetic animals and markedly improved their general condition. Collip introduced improvements in the method of preparation by showing that the crude insulin was soluble in concentrations of alcohol up to 80 per cent, above which it begins to precipitate.^{4, 9, 10} The marked lowering of the blood sugar of normal rabbits until 0.045 per cent is reached, when the animal goes into convulsions, was adopted as a basis of assaying the activity of extracts.

The Banting group was amazingly active during the years 1921–1923. They studied the effect of insulin on carbohydrate metabolism and showed that the respiratory quotient was definitely increased. They noticed its stimulating effect on the glycogenic function of the liver. They reported the disappearance from the urine of acetone bodies; these substances constitute one of the very bad effects of diabetes. They described the results of insulin treatment in 50 diabetic patients. All the patients were benefited, the results being particularly striking with children and young adults.¹¹

Sources of Insulin.—The main source is the islets of Langerhans of the mammalian pancreas. The islet tissue of the codfish has also proved a fruitful source of the hormone. Vegetable extracts have also been prepared which lower the blood sugar, but whether these extracts contain insulin is doubtful. Its presence in the urine has not so far been established.⁴

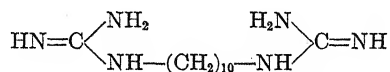
Methods of Preparation.—Improved methods of preparing insulin extracts have been suggested since Collip's pioneer work. Dudley, for example, obtained the hormone in the form of its picrate and hydrochloride. Shaffer extracts the gland with strongly acidified alcohol and precipitates the crude substance with ammonium sulfate.

Further purification is accomplished by adjusting the pH. Scott, following Shaffer's method rather closely, depends upon further purification by a final precipitation with trichloroacetic acid.

These various extracts, of varying degrees of purity, show insulin to be a protein-like substance with an isoelectric point around pH 5. It is destroyed by trypsin and is soluble in dilute acids and alkalis, being relatively stable in the former and unstable in the latter.⁴

The Chemistry of Insulin.—Abel and his associates have prepared insulin in the crystalline state, and it is now generally agreed that the hormone in this condition is probably chemically pure. The fundamental observation of Abel that crystallization of the active substance could be induced by alternately precipitating and extracting with pyridine and phenol, led to its final isolation.¹² The hydrolysis of the crystalline material has so far failed to yield anything but amino acids. These include cystine, tyrosine, arginine, histidine, lysine, leucine and glutamic acid. Tryptophan has not been detected. During the course of peptic hydrolysis of insulin, the decrease in physiologic activity is accompanied by a decrease in the tyrosine content.^{13, 56}

Synthalin.—This synthetic substance, a decamethylenediguanidine,



has been used, in a very limited way, as a substitute for insulin, because the latter is destroyed when given orally whereas synthalin is not. However, its toxicity and several other shortcomings have limited its use.⁴

Intarvin.—This synthetic odd-carbon fat was introduced in the attempt to avoid the formation of acetone bodies in diabetes. It, also, for various reasons, has not come into general use.⁴

THE PITUITARY HORMONES

The pituitary, situated at the base of the skull and weighing around 0.5 Gm., has a multiplicity of hormones to its credit, some of them far from being definitely identified. In the anterior portion there is but little doubt that hormones influencing sex, growth, the thyroid and carbohydrate metabolism, have been shown to be present; and in the posterior portion there are present, at least, oxytocic and pressor principles.

The Sex-stimulating Hormone of the Anterior Pituitary.^{14, 15}—Smith, Zondek and their collaborators showed how the transplantation of anterior pituitary tissue into sexually immature mice and rats induced precocious sexual maturity. "The results secured by anterior pituitary transplantations in the normal animal are harmonious with those secured from hypophysectomy and a replacement therapy. After hypophysectomy, the gonads degenerate. The large follicles become atretic. Pituitary transplants restore the gonads to a normal or

nearly normal size, causing a resumption of follicular growth and a reappearance of oestrus in the female, and induced spermatogenesis in the male."^{16, 17} Since then, active extracts have been prepared from the pituitary, and a hormone in some way similar to the pituitary hormone has been obtained from the pregnant urine of the female.

The methods of preparation vary considerably. In some cases alkaline extracts are made; in others, pyridine extracts; in still others, extracts with sodium acetate-acetic acid buffer (*pH* 4.5). The separation from other hormones depends upon subsequent fractionations.⁴

Fevold and Hisaw, using a pyridine extraction, find that they can prepare two active fractions: A water-soluble fraction which produces follicular development in the ovaries of sexually immature rats, and a relatively insoluble portion devoid of follicular effect. When the two fractions are united, a typical luteinized ovary is produced, indicating that the water-insoluble fraction stimulates luteinization when the follicles have been stimulated by the water-soluble hormone.^{18, 4} Wallen-Lawrence has confirmed the existence of these two hormones.¹⁹

Van Dyke has suggested the name *hebin* ("puberty") for the gonad-stimulating hormone.

The active material in this impure state is insoluble in organic solvents and is precipitated from aqueous solutions by alcohol and acetone. The hormone is also precipitated by saturated ammonium sulfate and several alkaloidal reagents.

Anterior Pituitary-like Sex Hormone in the Blood.—Zondek and Frank have shown the presence of this hormone in the blood of pregnant women.¹⁵

Hormone of the Intermediary Lobe of the Hypophysis.—By extracting the acetone-extracted dried powdered hypophysis with dilute acetic acid, Zondek obtained an extract which when injected into the minnow at a time other than the spawning period, causes the development of an intensive red coloration at the site of attachment of the pectoral, ventral and anal fins. (Only during the spawning period is the red coloration present.²⁰)

The Growth Hormone of the Anterior Pituitary.—Dwarfism and gigantism have long been associated with pituitary disorders. The hyperpituitary type, as shown by the acromegalic patient, has been particularly studied by Cushing.²¹ Active extracts, however, were first prepared by Evans,^{22, 23} using saline suspensions and, later, alkaline extracts. He produced experimental gigantism in rats and also provoked the growth of hypophysectomized puppies. Van Dyke, who suggested the name *phyone* ("I cause to grow") for the growth hormone, extracts the materials with 1 per cent sodium hydroxide, adjusts the *pH* values and fractionates with sodium sulfate.²⁴ Using female hypophysectomized rats, a potent extract will show an increase of 16 per cent of the total weight of the animal in four days. Active extracts have also been prepared by Collip.²⁵

Thyreotropic Hormone of the Anterior Pituitary.—Smith has shown that when the pituitary in rats is extirpated the thyroid atrophies; improvement is shown when fresh hypophysis is implanted into the hypophysectomized animal. Loeb has prepared an extract, which, when injected into animals, produced a marked hypertrophy of the thyroid closely resembling that seen in Graves' disease.²⁶ Marine has produced exophthalmos in the guinea-pig using acid extracts of ox anterior pituitary powder.²⁷ Krogh has shown the active material to be soluble in water and in 48 per cent alcohol, but insoluble in 70 per cent—observations which have helped in the purification.⁴

Hormone in Anterior Pituitary Involved in Carbohydrate (and Fat?) Metabolism.—We are indebted to Houssay for pioneer work in this field.²⁸ He showed that the injection of insulin in amounts not harmful to the normal dog would bring about coma and death in the hypophysectomized animal. In a study on toads, he showed that whereas pancreatectomy produces an intense diabetes, if the pituitary is removed, pancreatectomy does not give rise to diabetes. However, the implantation of an anterior lobe beneath the skin develops diabetes in the animal.

Anselmino has prepared fractions from the anterior pituitary, which, when injected into rabbits, markedly increase the acetone bodies of the blood.²⁹ Funk has obtained a somewhat similar substance from urine.³⁰ To what extent this is an independent hormone, and to what extent it is related to the hormone influencing carbohydrate metabolism, is at present debatable.³¹

The Lactogenic Hormone of the Anterior Pituitary.¹⁴—Riddle has obtained an extract of the anterior pituitary which gives rise to the effective stimulus for the enlargement and functioning (formation of "crop-milk") of the crop-glands in pigeons.³² The names *prolactin* and *galactin*³³ have been given to this hormone.

Adrenaltropic Hormone of the Anterior Pituitary.—Ablation of the pituitary causes atrophy of the cortex of the adrenal. It has been claimed that improvement can be brought about by the injection of potent extracts; but the field has not yet been cleared up.²³ *

The Pregnancy Test.—Anterior pituitary-like substances have been prepared from the urine of pregnancy but it is doubtful whether this sex-stimulating hormone and the one derived from the pituitary are one and the same substance. It is not improbable that the placenta is the source of the hormone (or hormones?) in pregnancy urine. The pregnancy test we owe to Aschheim and Zondek.^{34, 15} Five immature mice, three to four weeks old, are injected with the urine, each mouse receiving six injections of 0.4 cc. of urine (a total of 2.4 cc.) in two days. On the fourth day the animals are killed and their ovaries are inspected for hemorrhagic spots ("Blutpunkte") and yellowish protrusions (developed corpora lutea). The presence of hemorrhage within

* For a recent review dealing with the complex field of anterior pituitary hormones, see⁵⁷. See also⁵⁸.

the follicles and the presence of corpora lutea in at least one of the animals tested indicate pregnancy in the patient. (There are a number of variations of this test.)

Concentrated extracts of this hormone found in the urine of pregnancy have been prepared by several investigators.⁴ One such method depends upon the adsorption of the active material on benzoic acid. Both the benzoic acid and the female hormone (which is also present) are removed by a fat solvent, the anterior pituitary-like hormone (the "pregnancy" hormone) not being soluble in fat solvents, but soluble in water.

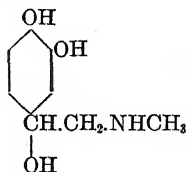
The Posterior Lobe of the Pituitary.—Aqueous extracts of this gland (*pituitrin*) produce various physiologic reactions (cardiovascular, plain muscle and melanophore stimulation, diuretic-antidiuretic, etc.). Kamm has separated two fractions, one an oxytocic, and the other a pressor principle.^{35, 4}

None of the hormones of the pituitary have so far been isolated in the chemically pure state.

THE ADRENAL HORMONES

In the medulla of the adrenal gland we find adrenaline (or epinephrine), which has not only been isolated but synthesized; and in the cortex of the gland there is a very active and, in some ways, even more important hormone called "cortin," which has not, as yet, been isolated.

Adrenaline.*—Schäfer in 1894 discovered that an extract of the adrenal glands caused a rise in blood pressure. Several workers busied themselves with the isolation of the active principle, among them Abel and Takamine.³⁶ In principle, the gland is extracted with acidulated water, the extract concentrated, inert material is removed with methyl alcohol, and the hormone is precipitated with ammonia. The formula, confirmed by synthesis, was shown to be



which shows it to be catechol with a hydroxyethylmethylamine group.

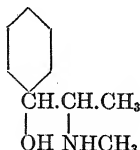
The *l*-form is fifteen times as active as the *d*-form. The physiologic assay—the pressor test—consists in comparing the effect on the blood pressure of an animal of a known (and standardized) sample with the unknown specimen.

Adrenaline constricts the blood vessels, thereby increasing the blood pressure; it stimulates the vagus center causing slowing of the heart; it has a stimulating effect on the heart muscle; it has a vasoconstricting action in hemorrhage; etc.

* Also called epinephrine.

It is believed, though with far from sufficient experimental evidence, that tyrosine may be the mother substance of adrenaline, just as it may be the forerunner of thyroxine.

Ephedrine.—This is a plant and not an animal product. But ephedrine and adrenaline are chemically and physiologically related. Ephedrine has the formula



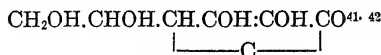
so that it is 1-phenyl-1-hydroxy-2-methylaminopropane. It is obtained from a Chinese plant, *Ephedra vulgaris*. All its four isomers have been prepared synthetically.

The Cortical Hormone.—Addison's disease, connected with a diseased cortex, cannot be cured with adrenaline. It is only within the last few years that an active cortical extract—called *cortin* by some—has been prepared.^{37, 38, 39, 59}

The average life-span of adrenalectomized cats is from seven to eight days. Extracts have been prepared which, when injected into such animals can apparently prolong their life to an age comparable with that of control animals. The extract is a lipid fraction of the gland which can be purified by the use of several lipid solvents. Kendall⁶⁰ has announced the isolation of an active crystalline compound, with the formula $\text{C}_{20}\text{H}_{30}\text{O}_5$.

A remarkable clinical application of the cortical hormone is in Addison's disease, which was described by its discoverer in 1855 as exhibiting in the patient "anemia, general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach, and a peculiar change of color of the skin."⁴ The cortical hormone apparently causes some striking recoveries.⁴⁰

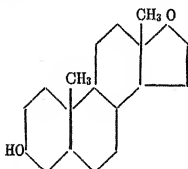
Ascorbic Acid.—This substance, already mentioned (pp. 66 and 303) was first isolated from the adrenal cortex. It is apparently the vitamin C (the antiscorbutic vitamin), with the formula



THE MALE HORMONE

From the testicle and from male urine, extracts have been obtained which control the secondary sexual characters in the male—"internal, such as the accessory reproductive organs, and external, such as horn growth and the growth of the cock's comb."⁴ The extracts are all lipoidal in character—that is, fat solvents are used for extraction and purification. A potent extract will restore the comb growth in a castrated cock.^{43, 44}

Butenandt has reported the isolation of this hormone. He gives to it the formula $C_{19}H_{30}O_2$ and is of the opinion that it is a saturated compound having, among other groups, a keto and a hydroxyl group.⁴⁵ He has related its structure to that of the female hormone (a phenanthrene derivative) and suggests the following formula:⁶⁸



This structure has recently been beautifully confirmed by Ruzicka,⁶¹ who oxidized a stereoisomer of dihydrocholesterol (*epidihydrocholesterol*) with chromic acid and obtained the male hormone (androst-4-en-3-one).

THE FEMALE HORMONES

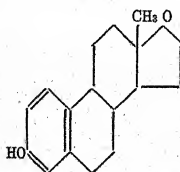
Stockard and Papanicolaou showed that "during the anabolic phase of the cycle of estrual changes, the epithelium of the vagina grows to a considerable thickness and a cornified layer similar to that in the epidermis develops. During the catabolic phase, the outer layers of this epithelium degenerate and are removed by leucocytic action. These changes provide a definite succession of cell types in the vaginal lumen, each one characteristic of a certain phase of the cycle. Thus the microscopical examination of vaginal smears is a reliable indicator of the estrual condition of the living animal."⁷⁴

Such cyclic changes cease when the animal (the rat, in this case) undergoes double ovariectomy. Allen and Doisy were the first to show that they could produce a potent extract which produced the phenomena of estrus in the ovariectomized animal.⁴⁶

The active material (called *theelin* by some, *estrin* by others, and a half dozen additional names by still others) may be obtained from fresh liquor folliculi; but a very convenient source is the urine of pregnant women and, still better, the urine of pregnant mares. In all cases, use is made of its solubility in fat solvents for extraction purposes.

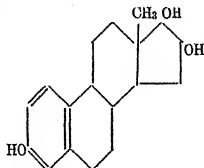
The true theelin or estrin, as well as several related compounds, has been isolated in the chemically pure form.^{47, 48, 49, 4}

Theelin has the formula $C_{18}H_{22}O_2$. It has a carbonyl and a hydroxyl group (the latter showing acidic properties) and is unsaturated. Its formula is probably



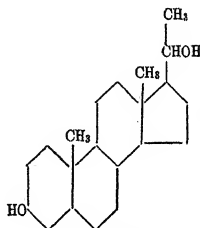
Theelin (or ketohydroxyestrin).

The presence of another compound, but less active physiologically, has also been isolated. It is called *theelol* and has the formula $C_{18}H_{24}O_3$. It forms a triacetate, indicating three hydroxyl groups and—as Butenandt showed—it can be converted to theelin by heating with potassium acid sulfate. Its probable formula is



Theelol (or trihydroxyestrin).

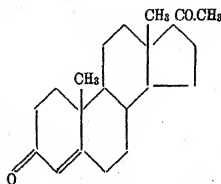
An inactive alcohol, pregnandiol, has also been isolated, to which the formula



Pregndiol.

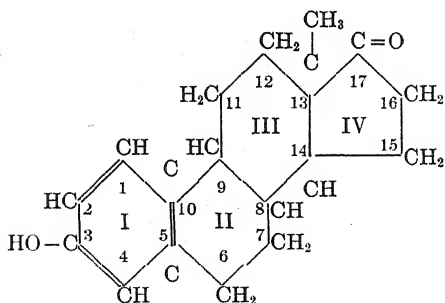
has been assigned.^{7, 4} This does not exhaust the number of phenanthrene derivatives the presence of which has been determined.

A corpus luteum hormone (*progestin*), isolated in the impure state by Corner and Hisaw, among others,⁴ which exerts a specific proliferative action on the uterine endometrium, has been obtained in the crystalline form.^{62, 63, 64} The formula suggested is



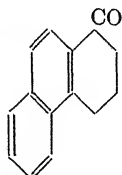
Butenandt⁶⁵ has obtained the substance by a degradation of the side chain of one of the sterols (stigmasterol).

The structure and numbering of these phenanthrene derivatives may be illustrated with the female hormone:



so that the systematic name for theelol would be 3,16,17-trihydroxy-1,3,5-estratriene. (In this connection, compare these structures with the sterols and bile acids (Chapter IV) and with the structure of ergosterol (p. 286).)*

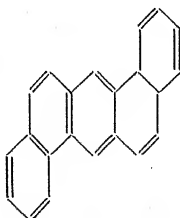
Synthetic Products.—It has been shown by Cook and Dodds that several synthetic compounds, related to theelin, have estrus-like properties. For example, 1-keto-1: 2: 3: 4-tetrahydrophenanthrene



(A)

is a compound of this type.

Even greater activity has been shown among compounds derived from 1: 2: 5: 6-dibenzanthracene

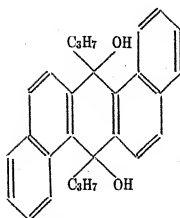


(B)

each half of the molecule of which is of the type of (A).⁷ Here one compound in particular has been found to be more active physiologically than theelol.

Suggestive, indeed, is the fact that the carcinogenic substances present in tar are related to the hydrogenated phenanthrene ring. "The most potent synthetic estrus-producing agent is 9: 10-di-*n*-propyl-9-10-dihydroxy-1: 2: 5: 6-dibenzanthracene:

* See also the excellent review by Rosenheim and King.⁶⁶



which is a derivative of (B), the carcinogenic hydrocarbon.”⁷ Furthermore, vitamin D, which is also a derivative of phenanthrene (see p. 286) has been shown to have estrogenic activity.

SECRETIN

Bayliss and Starling were the first to show that a hormone controlled the flow of pancreatic juice into the small intestine.⁵⁰ To this hormone they gave the name *secretin*. The active extract may be obtained from the upper two thirds of the small intestine by a process which involves extraction with dilute hydrochloric acid. The hormone has so far not been isolated.

It has been shown by Ivy that a hormone other than secretin, but commonly associated with it, is primarily responsible for the stimulation of the hepatic cells, augmenting the flow of bile. Ivy calls this substance *cholecystokinin*.⁵²

Mention may here be made of attempts to show the presence of a *gastrin*—a hormone involved in the flow of gastric juice; but the results are far from decisive, some believing that whatever hormone action is obtained is due to histamine.⁵¹

PLANT HORMONES

Kögl has isolated a substance which affects curvature (cell-stretching) in plants.^{53, 4} It may be obtained from the tips of oats and corn, from yeast and also from human urine (which, by the way, is the most convenient source). The material is extracted from acid media with organic solvents. *Auxin*—the name given to the hormone—has the formula C₁₁H₃₂O₅. The substance contains three hydroxyl groups and one double bond.

Butenandt has isolated in the chemically pure form a substance from the kernel of the palm tree (*tokokinin*) which he has shown to be identical with the female hormone.⁵⁴ Apparently, plant hormones can be found in animal products and *vice versa*.*

BENJAMIN HARROW.

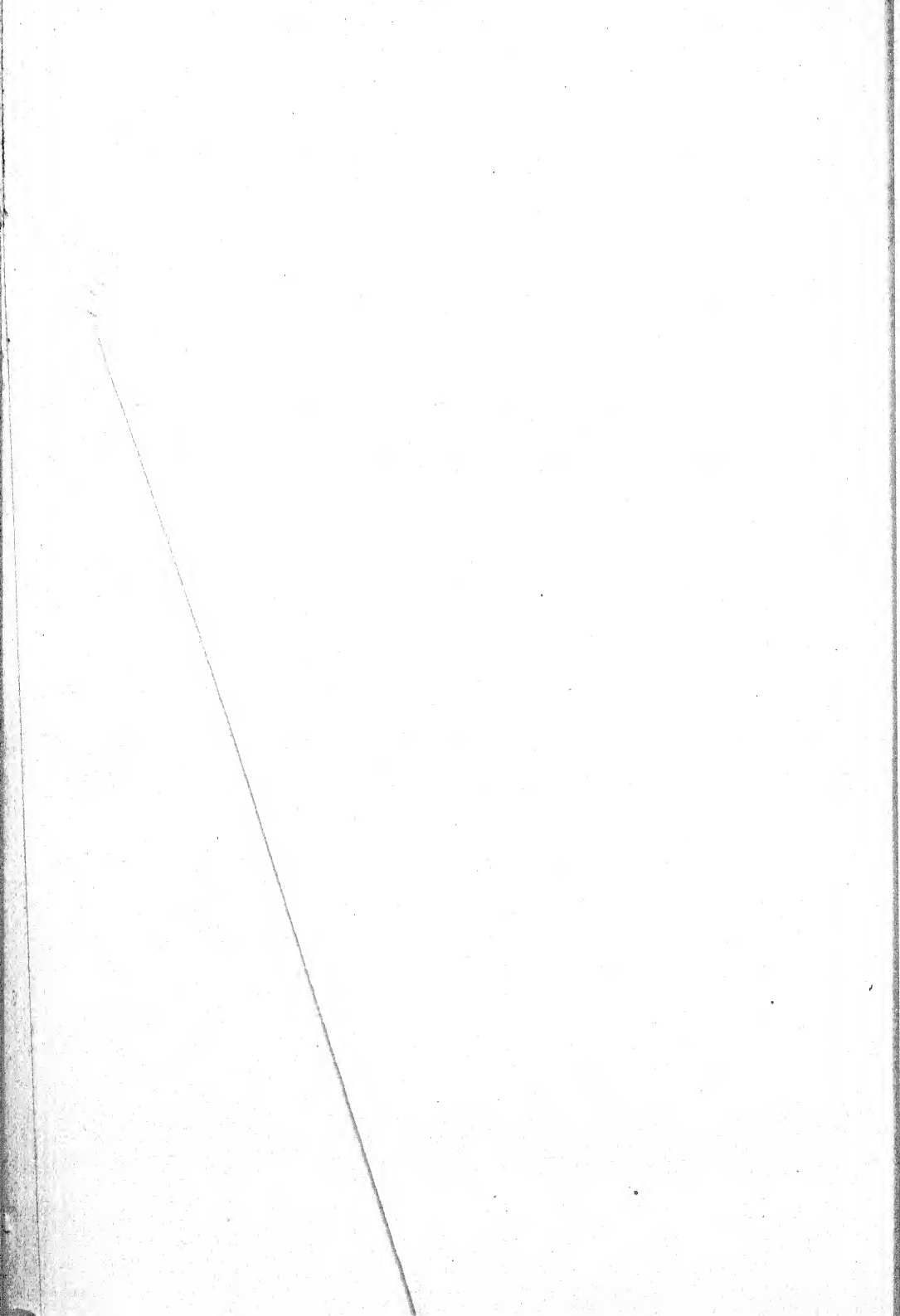
REFERENCES

1. Heidelberger, M., and Palmer, W. W.: *J. Biol. Chem.*, 101, 433 (1933).
2. Kendall, E. C.: *J. Biol. Chem.*, 39, 125 (1919).
3. Harington, C. R.: *The Thyroid Gland* (1933).

* See also ⁶⁷.

4. Harrow, B., and Sherwin, C. P.: *The Chemistry of the Hormones* (1934).
5. Foster, G. L.: *J. Biol. Chem.*, **83**, 345 (1929).
6. Cameron, A. T.: *Recent Advances in Endocrinology* (1934).
7. Dodds, E. C.: *Lancet*, **1**, 931, 987, 1048 (1934).
8. Collip, J. B.: *J. Biol. Chem.*, **63**, 395 (1925); Thomson, D. L., and Collip, J. B.: *Physiol. Rev.*, **12**, 309 (1932).
9. Banting, F. G., and Best, C. H.: *J. Lab. Clin. Med.*, **7**, 251 (1922).
10. Banting, F. G., Best, C. H., Collip, J. B., Macleod, J. J. R., Noble, E. C., and Hepburn, J.: *Trans. Roy. Soc. Canada*, **16**, 1 (1922).
11. Macleod, J. J. R.: *J. Am. Med. Assoc.*, **80**, 1238, 1847 (1923); *Physiol. Rev.*, **4**, 21 (1924).
12. Abel, J. J., and Geiling, E. M. K.: *J. Pharmacol.*, **25**, 423 (1925).
13. Jensen, H., and Evans, E. A., Jr.: *Physiol. Rev.*, **14**, 188 (1934).
14. Allen, E.: *Sex and Internal Secretions* (1932).
15. Mazer, C., and Goldstein, L.: *Clinical Endocrinology of the Female* (1932).
16. Smith, P. E., and Engle, E. T.: *Am. J. Anat.*, **40**, 159 (1927).
17. Zondek, B., and Aschheim, S.: *Klin. Wochschr.*, **6**, 248 (1927).
18. Fevold, H. L., Hisaw, F. L., and Leonard, S. L.: *Am. J. Physiol.*, **97**, 291 (1931).
19. Wallen-Lawrence, Z.: *J. Pharmacol.*, **51**, 263 (1934).
20. Zondek, B., and Krohn, H.: *Klin. Wochschr.*, **11**, 1293 (1932).
21. Cushing, H.: *Harvey Lectures* (1934), p. 90.
22. Evans, H. M., and Long, J. A.: *Anat. Record*, **21**, 62 (1921).
23. Evans, H. M.: *J. Am. Med. Assoc.*, **101**, 425 (1933).
24. Van Dyke, H. B., and Wallen-Lawrence, Z.: *J. Pharmacol.*, **40**, 413 (1930).
25. Collip, J. B., Selye, H., and Thomson, D. L.: *Proc. Soc. Exptl. Biol. Med.*, **30**, 544 (1933).
26. Loeb, L., and Bassett, R. B.: *Proc. Soc. Exptl. Biol. Med.*, **26**, 860 (1929).
27. Marine, D., and Rosen, S. H.: *Proc. Soc. Exptl. Biol. Med.*, **30**, 901 (1933).
28. Houssay, B. A., and Biasotti, A.: *Endocrinology*, **15**, 511 (1931).
29. Anselmino, K. J., and Hoffmann, F.: *Klin. Wochschr.*, **10**, 2380 (1931).
30. Funk, C.: *Proc. Am. Soc. Biol. Chem.*, **8**, 43 (1933).
31. Harrow, B.: *Science*, **79**, 272 (1934); Harrow, B., Naiman, B., Chamelin, I. M., and Mazur, A.: *Proc. Soc. Exptl. Biol. Med.*, **31**, 940 (1934); *Am. J. Physiol.*, **109**, 436 (1934).
32. Riddle, O., Bates, R. W., and Dykshorn, S. W.: *Am. J. Physiol.*, **105**, 191 (1933).
33. Gardner, W. U., and Turner, C. W.: *Mo. Agric. Exp. Sta., Research Bull.*, **196**, 5 (1933).
34. Aschheim, S., and Zondek, B.: *Klin. Wochschr.*, **7**, 1401 (1928).
35. Bugbee, E. P., and Kamm, O.: *Endocrinology*, **12**, 671 (1928).
36. Abel, J. J.: *Johns Hopkins Hosp. Bull.*, **13**, 29 (1902); Takamine, J.: *Am. J. Pharm.*, **73**, 523 (1901).
37. Rogoff, J. M., and Stewart, G. N.: *J. Am. Med. Assoc.*, **92**, 1569 (1929); **99**, 1309 (1932).
38. Swingle, W. W., and Piffner, J. J.: *Am. J. Physiol.*, **96**, 164 (1931); *Endocrinology*, **15**, 335 (1931).
39. Hartman, F. A., Brownell, K. A., and Hartman, W. E.: *Am. J. Physiol.*, **95**, 670 (1930).
40. Rowntree, L. G., and Ball, R. G.: *Endocrinology*, **17**, 263 (1933).
41. Svirbely, J. L., and Szent-Györgyi, A.: *Biochem. J.*, **27**, 279 (1933).
42. Hawarth, W. N., Hirst, R., et al.: *J. Chem. Soc.*, p. 1419 (1933).
43. Gallagher, T. F., and Koch, F. C.: *J. Biol. Chem.*, **84**, 495 (1929); Koch, F. C.: *J. Am. Med. Assoc.*, **96**, 937 (1931).
44. Funk, C., Harrow, B., and Lejwa, A.: *Am. J. Physiol.*, **92**, 440 (1930); Funk, C., and Harrow, B.: *Biochem. J.*, **24**, 1678 (1930).
45. Butenandt, A.: *Z. angew. Chem.*, **45**, 655 (1932); *Naturwissenschaften*, **21**, 49 (1933); Tscherning, K.: *Ergebnisse Physiol.*, **35**, 301 (1933).
46. Allen, E., and Doisy, E. A.: *J. Am. Med. Assoc.*, **81**, 819 (1923).
47. Doisy, E. A., Veler, C. D., and Thayer, S.: *J. Biol. Chem.*, **86**, 499 (1930).
48. Marrian, C. F.: *Physiol. Rev.*, **13**, 185 (1933).
49. Butenandt, A.: *Z. physiol. Chem.*, **191**, 127 (1930).
50. Bayliss, W. M., and Starling, E. H.: *J. Physiol.*, **29**, 174 (1902).
51. Sacks, J., Iry, A. C., Burgess, J. P., and Vaudolah, J. E.: *Am. J. Physiol.*, **101**, 331 (1932).

52. Ivy, A. C., Kloster, G., Lueth, H. C., and Drewer, G. E.: *Am. J. Physiol.*, **91**, 336 (1929).
53. Kögl, F.: *Naturwissenschaften*, **21**, 17 (1933).
54. Butenandt, A., and Jacobi, H.: *Z. physiol. Chem.*, **218**, 104 (1933).
55. Jones, J. H.: *J. Biol. Chem.*, **106**, 701 (1934); Taylor, N. B., Weld, C. B. and Sykes, J. F.: *Proc. Roy. Soc. (London)*, **116B**, 10 (1934); Thomson, D. L., and Collip, J. B.: *International Clinics*, **4**, 103 (1933).
56. Fisher, A. M., and Scott, D. A.: *J. Biol. Chem.*, **106**, 289 (1934).
57. Collip, J. B.: *J. Mount Sinai Hospital*, **1**, 28 (1904).
58. Thomson, D. L., and Collip, J. B.: *Annual Rev. Biochem.*, **3**, 225 (1934).
59. Pfiffner, J. J., Swingle, W. W., and Vars, H. M.: *J. Biol. Chem.*, **104**, 701 (1934).
60. Kendall, E. C., Mason, H. L., McKenzie, B. F., and Myers, C. S.: *Proc. Staff Meetings Mayo Clinic*, **9**, 245 (1934).
61. Ruzicka, L., Goldberg, M. W., Meyer, J., Brüngger, H., and Eichenberger, E.: *Helv. Chim. Acta*, **17**, 1389, 1395 (1934).
62. Slotta, K. H., Ruschig, H., and Blanke, E.: *Ber.*, **67**, 1947 (1934).
63. Butenandt, A., Westphal, U., and Hohlweg, W.: *Z. physiol. Chem.*, **227**, 84 (1934).
64. Wintersteiner, O., and Allen, W. M.: *J. Biol. Chem.*, **107**, 321 (1934).
65. Butenandt, A., Westphal, U., and Cobler, H.: *Ber.*, **67**, 1611 (1934).
66. Rosenheim, O., and King, H.: *Annual Rev. Biochem.*, **3**, 87 (1934).
67. Thimann, K. V., and Bonner, J.: *Proc. Roy. Soc. (London)*, **113B**, 16 (1933).
68. Butenandt, A., and Tscherning, K.: *Z. physiol. Chem.*, **229**, 167, 185, 192 (1934).



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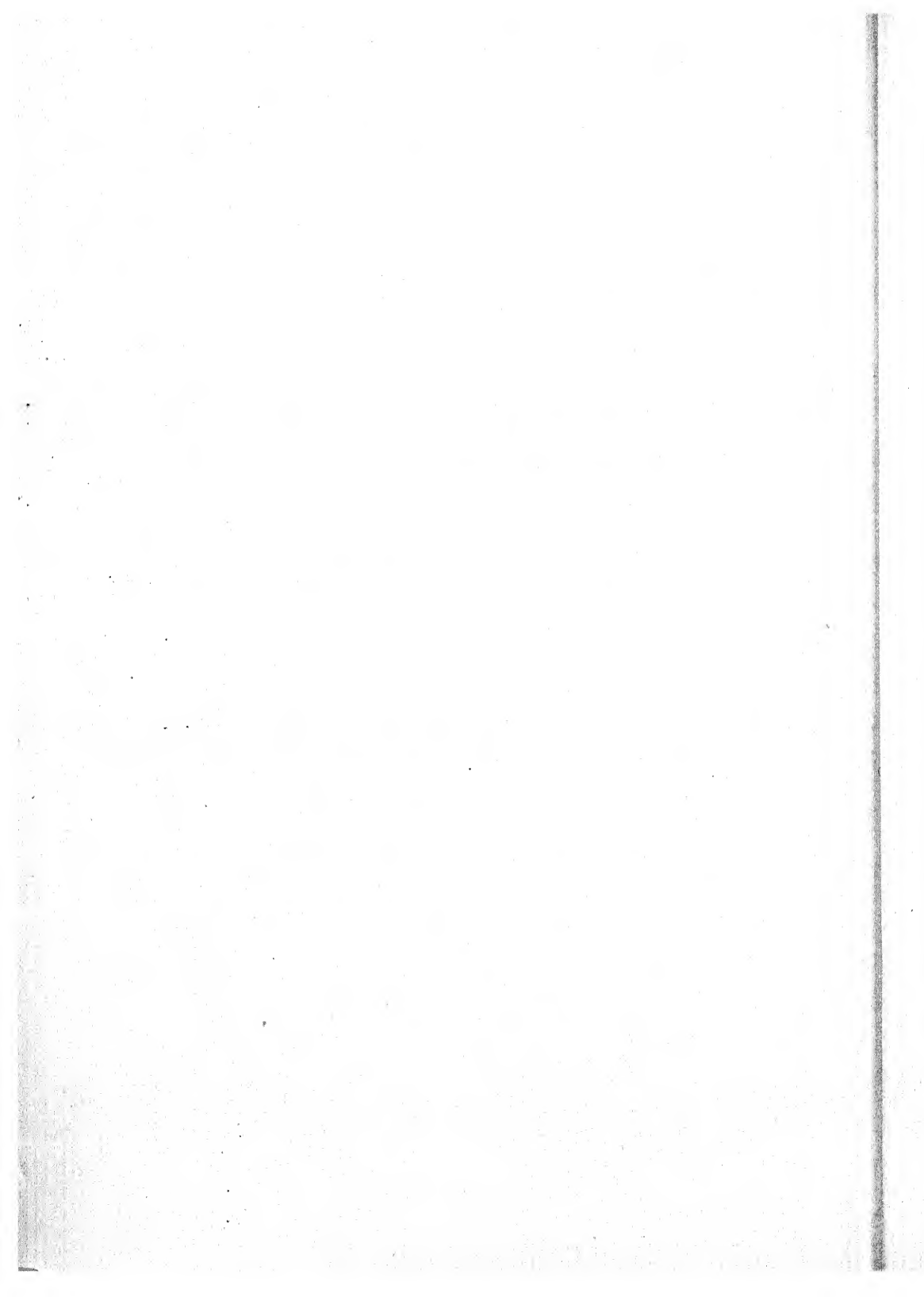
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